

The anti-CD20 mAb LFB-R603 interrupts the dysregulated NF- κ B/Snail/RKIP/PTEN resistance loop in B-NHL cells: Role in sensitization to TRAIL apoptosis

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Abstract. LFB-R603 is a chimeric anti-CD20 mouse/human monoclonal antibody with a human IgG1 constant (Fc) region. In comparison to rituximab, LFB-R603 exhibits a high affinity to the Fc γ RIII (CD16) and a potent *in vitro* antibody-dependent cellular cytotoxicity (ADCC). We examined several experimental designs for the biological anti-tumor activity of LFB-R603 as well as its sensitizing activity to apoptosis in resistant non-Hodgkin's B-cell lymphoma (B-NHL) in comparison to rituximab. Treatment of the B-NHL cell line Ramos with LFB-R603 was not toxic at the concentrations used and induced cell aggregation following culture at 24 and 48 h similarly to rituximab. Hence, we hypothesized that LFB-R603 may signal the tumor cells and modify intracellular survival/anti-apoptotic pathways. Treatment of Ramos cells with LFB-R603 inhibited the constitutively active NF- κ B survival pathway, followed by significant potentiation of TRAIL-mediated apoptosis. We examined the underlying molecular mechanism by which the LFB-R603-mediated NF- κ B inhibition results in

the reversal of tumor cell resistance to TRAIL. We hypothesized that downstream gene products regulated by NF- κ B that are involved in the resistance may be involved in LFB-R603-mediated sensitization. We found that LFB-R603 inhibited NF- κ B activation and the anti-apoptotic factor Snail and induced the pro-apoptotic factor RKIP. The direct roles of Snail and RKIP modulation by LFB-R603 in the reversal of B-NHL resistance to TRAIL were examined by knocking down Snail and overexpressing RKIP in Ramos cells, respectively. Both approaches increased significantly the cell sensitivity to TRAIL apoptosis. In addition to changes observed in the expression levels of Snail and RKIP, Ramos cells treated with LFB-R603 induced the expression of PTEN along with inhibition of the PI3K-AKT activated pathway. PTEN silencing in Ramos cells pretreated with LFB-R603 and TRAIL inhibited TRAIL apoptosis; thus, demonstrating that PTEN induction by LFB-R603 has a direct role in tumor cell sensitization to TRAIL apoptosis. Several of the findings obtained in the experimental designs with LFB-R603 were superior to those obtained with rituximab. Overall, the findings demonstrate that LFB-R603 interferes with the dysregulated NF- κ B/Snail/RKIP/PTEN/AKT resistance circuitry in B-NHL cells. Further, the findings suggest that LFB-R603 may sensitize tumor cells to various apoptotic stimuli including cytotoxic ligands such as TRAIL and chemotherapeutic drugs.

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Abbreviations: DHMEQ, dehydroxymethylepoxyquinomicin; ERK, extracellular signal-regulated kinase; IAPs, inhibitors of apoptosis proteins; KD, dissociation constant; K_{off}, dissociation rate constant; K_{on}, association rate constant; MAPK, mitogen-activated protein kinases; MEK, MAP kinase kinase; NF- κ B, nuclear factor- κ B; TRAIL, tumor necrosis factor-related apoptosis-inducing factor

Key words: apoptosis, LFB-R603, NF- κ B, PTEN, rituximab, RKIP, Snail, TRAIL

Introduction

B-cell non-Hodgkin's lymphoma (B-NHL) includes a number of clinicopathologic subsets of B-cell related neoplasms that have heterogeneous features based on clinical manifestation, prognosis and response to therapy (1-3). Diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) are the two most commonly diagnosed subtypes of NHL, accounting for approximately 60-70% of all NHLs in adults. DLBCL and Burkitt's lymphoma (BL) are also the most aggressive histological subtypes of B-NHL (4,5). The prognosis for NHL malignancies is strictly related to the histological subtype, tumor characteristics

and the host responses to treatment (6). The standard treatment for most NHLs is CHOP, a therapeutic scheme which is based in the combination of the chemotherapeutic regimens, cyclophosphamide (Cytosan) doxorubicin (hydroxydaunorubicin), vincristine (Oncovin) and one steroid known as prednisone. Administration of CHOP has a good prognosis with 45-50% of treated NHL patients surviving more than five years (7). An expanded protocol, called CHOP-R, has improved survival and rates of complete responses particularly for DLBL patients (8). R-CHOP is based on the concurrent use of CHOP with one anti-CD20 antibody, namely, rituximab (rituxan) (9,10). Rituximab is a chimeric monoclonal antibody against the protein CD20, which is primarily found on the surface of B cells (11,12). The demonstrated superior activity of rituximab in NHL patients' prognosis established the utility of the CHOP-R chemoimmunotherapy as the standard treatment in young and elderly NHL patients (13,14).

Although we still do not fully understand the mechanisms of action responsible for rituximab's anti-tumor effects *in vivo*, it is believed that complement-mediated cytotoxicity (CMC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) all appear to contribute to the clinical efficacy of rituximab (15-17). In addition, several studies have suggested that rituximab activity is dependent on CD20 expression for direct killing activity via CD20 signaling e.g., programmed cell death (PCD) and sensitization of cells to chemotherapy (15,18). Lymphoma cell survival depends on disruption of the apoptosis pathway by mutations in apoptosis-inducing genes or by continuous expression of anti-apoptotic proteins. Rituximab has been shown to target these apoptosis inhibitors and enhance pro-apoptotic signals (19-23). Another reported mechanism of rituximab-mediated NHL sensitization to apoptosis to cytotoxic ligands such as TRAIL is via upregulation of death receptors on the cancer cell surface, thus implicating a novel *in vivo* role of host involvement in rituximab-mediated effects (24-26). We and others have reported that rituximab effects in inducing apoptotic signals in lymphoma cell lines might be mediated via upstream inhibition of constitutively activated survival signaling pathways (Raf-1-MEK1/2-ERK1/2, NF- κ B, and Akt), resulting in inhibition of cell growth by selective upregulation of anti-apoptotic gene products (21,26-28).

NF- κ B is constitutively activated in the majority of cancers including NHL and it is clearly associated with acquisition of tumor resistance to therapy (28,29). NF- κ B is the direct transcriptional activator of several anti-apoptotic gene products including IAPs, Bcl-xL and Bcl-2 and its inhibition by various chemical inhibitors or gene silencing methods has resulted in increased tumor sensitivity to apoptosis induced by chemotherapy or immunotherapy (28,30). Upon treatment with rituximab, there is downstream inhibition of the NIK/IKK/I κ B α /NF- κ B pathway resulting in inhibition of Bcl-xL and cIAPs transcription and in reversal of drug resistance in NHL (20,21). High NF- κ B activity has also been associated directly or indirectly with decreased expression of death receptors on the cancer cell surface, thus making cancer cells unresponsive to apoptotic signals mediated by cytotoxic ligands (31-33). Treatment of NHL cell lines with rituximab resulted in increased expression of DR5 and potentiation of apoptosis induced by TRAIL (26). In addition, NF- κ B activates the

transcription of gene products that have been indirectly linked to acquisition of tumor resistance to both chemotherapy and immunotherapy such as Snail (34-36). Snail silencing *in vitro* in cancer cell lines has been reported to enhance tumor apoptotic response to chemotherapy and TRAIL (35,37). One of the gene products that participates in the endogenous regulation of NF- κ B and Raf-1/MEK/ERK activities is the Raf-1 kinase inhibitor protein (RKIP) (38,39). RKIP inhibits the nuclear translocation of NF- κ B via elimination of the I κ B α phosphorylation by upstream kinases (38). NF- κ B, in turn, negatively regulates RKIP expression via inducing the transcriptional repressor of RKIP, Snail (40). RKIP has a direct role in enhancing tumor sensitization to TRAIL-mediated apoptosis by inhibiting TRAIL resistance-related signals derived from the activation of NF- κ B and its downstream targets such as the DR5 transcriptional repressor, Yin Yang 1 (YY1) and by upregulating DR5 expression on the cancer cell surface (33). Given the anti-survival role of RKIP, RKIP expression is significantly diminished in several tumors and cancer cell lines including B-NHL lines and its expression is inversely correlated with Snail expression (40-42). Treatment of B-NHL cell lines with rituximab induces a rapid increase in RKIP expression, thus eliminating NF- κ B activity in tumor cells (43).

We have previously shown that treatment of B-NHL cell lines with rituximab significantly inhibited the constitutively activated PI3K/Akt signaling pathway and this inhibition reversed tumor chemoresistance (27). The PI3K/Akt pathway is involved in different cellular functions such as growth, migration, survival and differentiation. It has been reported to be constitutively activated in several tumors including NHL (44). AKT, a serine/threonine protein kinase, is a key effector of the PI3K signaling pathway: activated AKT inhibits apoptosis and increases cell survival by phosphorylating the pro-apoptotic Bcl-2 family member Bad, caspase-9, human double minute 2 (HDM2), p53, FKHL1 and I κ B kinase (IKK) (45). PTEN (phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor, acts as a negative regulator to inhibit AKT activation. However, PTEN is frequently mutated or deleted in many human cancers, including NHL, thus leading to overactivation of the PI3K/AKT signaling pathway (46,47). PTEN is indirectly under the negative regulation of NF- κ B since NF- κ B functions as an activator of its transcriptional repressor, Snail (48). PI3K enhances NF- κ B activity via IKK α phosphorylation, thus amplifying the survival signals in cancer cells (49). Like NF- κ B, the constitutive activation of the PI3K/AKT pathway in NHL has been shown to play a critical role in acquisition of NHL-resistance to chemotherapy (50).

Although the beneficial contribution of rituximab in the treatment of B-NHL has been reported, there is a substantial number of patients (approximately 50%) who are refractory or develop resistance in the course of prolonged treatment with rituximab as single agent or in combination with CHOP (51). Therefore, improvements in therapeutic options for NHL treatment remain necessary. Novel anti-CD20 mAbs are in development that may enhance mAb therapy (52). LFB-R603 is a chimeric mouse/human mAb with a human IgG1 constant (Fc) region recognizing the B-cell antigen CD20 (53). Due to a specific pattern of glycosylation, characterized by low fucose content in its Fc region, LFB-R603 has an increased

affinity for the Fc γ RIIIA (CD16) and induces a stronger *in vitro* ADCC activity against B-cell when compared to rituximab. Reported findings demonstrate that LFB-R603 mediates a significant ADCC activity against CLL (54,55).

Given the superior activity of LFB-R603 and the resistance of a subset of NHL patients to rituximab treatment, we hypothesized that LFB-R603 may have an augmented anti-tumor activity in NHL cells via cell signaling and modifying intracellular survival/anti-apoptotic pathways. The present study was designed to test this hypothesis using the B-NHL cell line, Ramos, as model. The following aspects were investigated: i) Does LFB-R603 inhibit cell proliferation and induce cell aggregation in tumor cells? ii) Does LFB-R603 trigger the cells and inhibit the constitutively activated NF- κ B pathway? iii) Does inhibition of NF- κ B by LFB-R603 sensitize the TRAIL-resistant Ramos cells to TRAIL apoptosis? iv) Are the NF- κ B targeted gene products involved in resistance such as Snail and RKIP modified by LFB-R603 treatment of NHL and do they regulate tumor resistance to TRAIL apoptosis? v) Does the LFB-R603-mediated inhibition of the PTEN-repressor Snail result in the induction of PTEN? and vi) Does the induction of PTEN by LFB-R603 participate in the reversal of resistance to TRAIL? Several of the findings obtained in the above experimental designs with LFB-R603 were compared to those derived after cell treatment with rituximab. Our findings concur with the above hypothesis and establish LFB-R603 as a modifier of the dysregulated NF- κ B/Snail/RKIP/PTEN/AKT resistance circuitry in B-NHL and results in the reversal of resistance to apoptotic stimuli.

Materials and methods

Cell lines and reagents. The CD20⁺ human Burkitt's B-NHL cell line and the PC-3 prostate adenocarcinoma cell line were originally purchased from the American Type Culture Collection (ATCC) (Bethesda, MD). The cells were cultured in RPMI-1640 growth medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotics. Cells were maintained in a control atmosphere incubator at 37°C with saturated humidity and 5% CO₂. Rituximab was obtained commercially from Biogen Idec Inc., and Genentech Inc. (San Francisco, CA). LFB-R603 was obtained from the Laboratoire Francais du Fruntionnement et des Biotechnologies (LFB) (Les Ulis, France). Recombinant TRAIL was purchased from Peprotech (Rocky Hill, NJ). Rabbit anti-human p65, phospho-p65 (Ser536), Akt, phospho-Akt (Ser473) and PTEN antibodies, were purchased from Cell Signaling (Danvers, MA). The anti-human RKIP, Snail, β -actin and PTEN antibodies were obtained from Zymed (San Francisco, CA), Abcam (Cambridge, MA) and Chemicon (Temecula, CA), respectively. RPE-conjugated anti-human IgG antibody was purchased from Southern Biotech (Birmingham, AL). The secondary anti-mouse and anti-rabbit IgG-HRP antibodies as well as the small Interfering (si) RNAs against Snail, PTEN and RKIP were obtained from Santa Cruz, (Santa Cruz, CA). The HA-CMV-RKIP expression vector and the corresponding control empty plasmid (HA-CMV-EV) are a generous gift from Dr K.C. Yeung (University of Toledo, OH). The NF- κ B, DHMEQ, was provided by Dr Kazuo Umezawa (Keio University, Japan).

Analysis of cell viability and aggregation. Cell viability after cell treatment with various concentrations of rituximab or LFB-R603 for 24 or 48 h was assessed microscopically using the trypan blue dye exclusion method. Cell aggregation was observed visually under the microscope and the fields were photographed.

Antibody binding assay. The binding of rituximab and LFB-R603 on CD20 antigen expressed on the surface of Ramos cells was detected by flow cytometry. The cells were first incubated with 10 or 20 μ g/ml of either of the 2 anti-CD20 antibodies or human serum for 24 h. After thorough washing, the cells were labeled for 30 min at RT with a secondary RPE-conjugated anti-human IgG antibody and analyzed in a Flow Epics XL-MCL (Coulter). Antibody binding efficiency was recorded as mean fluorescence intensity (MFI).

Determination of apoptosis. Ramos cells were incubated in complete medium with various concentrations of rituximab or LFB-R603 for 24 h, followed by incubation with 2.5, 5 or 10 ng/ml TRAIL for 18 more hours. Forty-eight hours post-initial treatment the cells were harvested and apoptosis was assessed by flow cytometry using intracellular active caspase-3 staining as previously described (35). Population data were acquired on an Epics XL-MCL (Coulter) flow cytometry equipment and percentages of apoptotic cells were determined using the System II software.

Western blot analysis. Ramos cells were incubated in complete medium with various concentrations of rituximab or LFB-R603 for 24 h. Protein expression was analyzed by Western blot analysis. Total protein lysates were derived after cell lysis in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA). Total protein was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Total protein lysates (30 mg) were loaded and separated in 12% SDS-Page at 70 V. Protein transfer to nitrocellulose membrane Hybond ECL (Amersham Biosciences, Sunnyvale, CA) was performed overnight using the Trans-Blot SD semidry transfer cell system (Bio-Rad). The membranes were blocked in 5% dry milk in TBS-Tween for 1 h, followed by overnight incubation with 1/1,000 dilution of the primary antibodies for p65, phospho-p65, RKIP, Snail, PTEN, Akt and phospho-Akt. After thorough washing, membranes were incubated with the appropriate HRP-conjugated antibody at 1/5,000 dilution for 1 h and blots were developed by LumiGlo reagent containing peroxide substrate (Cell Signaling Technology).

Application of small interfering RNA (siRNA). Silencing of Snail and PTEN expression using specific siRNAs was performed using the TransIT-siQuest transfection reagent (Mirus Bio LLC, Madison, WI). According to the manufacturer's instructions, Ramos cells were plated in 0.5 ml of complete growth medium per well, at a density of 1-1.5x10⁶ cells per well one day before transfection. Transfection was performed the following day by adding to each well 200 μ l of Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) which contained a mix of 3 μ l TransIT-siQuest transfection reagent and siRNA to a final concentration of 25 nM per well. For the Snail knock-down experiments, the transfected cells were

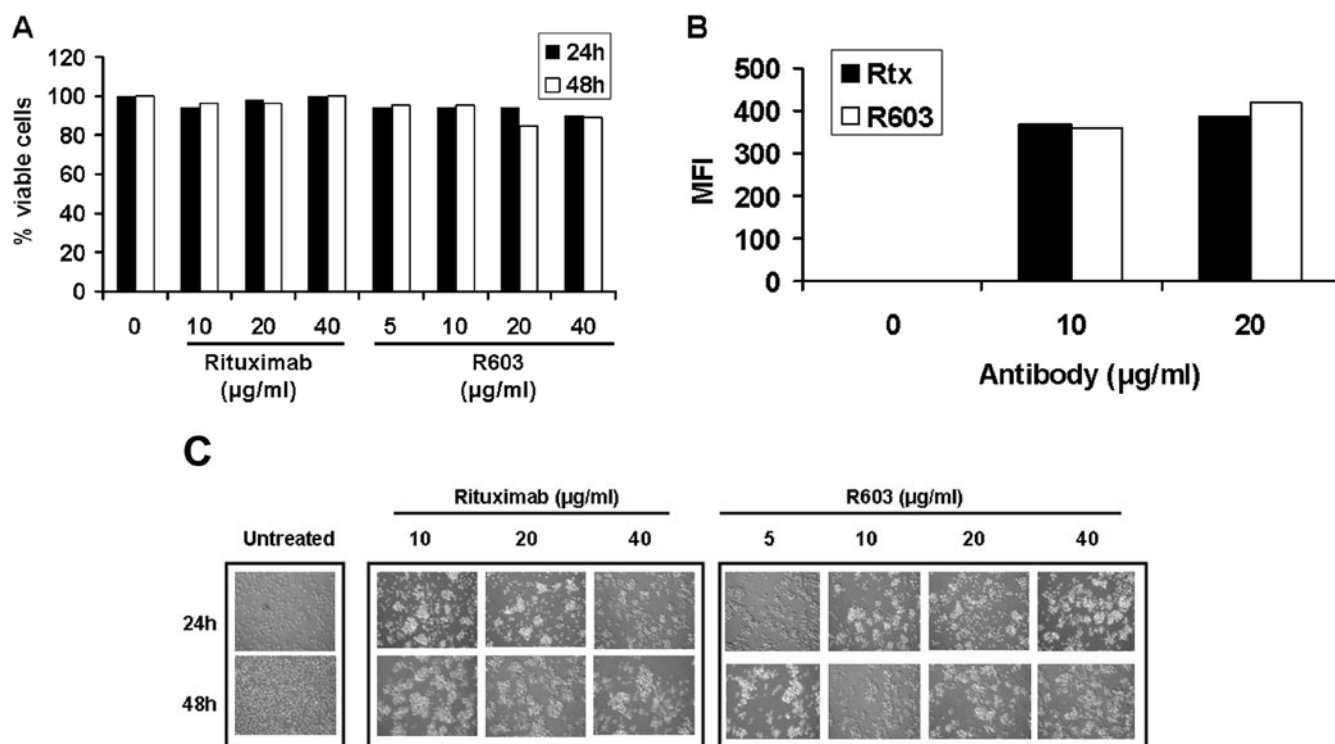


Figure 1. Biological activity of LFB-R603⁺ in comparison with rituximab. (A) Viability of Ramos cells treated with various concentrations of LFB-R603 or rituximab for 24 or 48 h in complete medium. The cells were analyzed for viability using the trypan blue dye exclusion. (B) Surface binding of LFB-R603 and rituximab on CD20⁺ Ramos cells. Ramos cells were treated with different concentrations of LFB-R603 or rituximab in complete medium for 24 h. Cells were washed and incubated for 30 min with a secondary RPE-labeled anti-human IgG antibody. The cells were analyzed by flow cytometry and the mean fluorescence intensity (MFI) of rituximab and LFB-R603 binding was calculated. (C) Induction of Ramos cell aggregation by LFB-R603 or rituximab. Cells were treated with LFB-R603 or rituximab as described above. Cell aggregate formation was examined microscopically and photographed (magnification x40). *For simplicity LFB-R603 in the figures is referred as R603.

incubated for 56 h followed by addition of 10 ng/ml TRAIL for further 24 h. For the PTEN knock-down experiments, 20 µg of LFB-R603 or rituximab was added to the cells 48 h post-transfection and incubated for 18 h. At the end of the 18 h, cells were further treated with 10 ng/ml TRAIL for further 24 h. In both experimental designs, cells were harvested and analyzed for apoptosis detection. The inhibition of Snail and PTEN protein expression after cell transfection with the corresponding siRNAs was verified by Western blot analysis.

Transient cell transfection with DNA vectors. For technical reasons related to difficulties in transfecting non-adherent cells with DNA plasmids, ectopic expression of RKIP was performed in the prostate adenocarcinoma cell line PC-3. PC-3 cells were transfected with CM-HA-RKIP vector or the corresponding control plasmid (CMV-HA-EV) in a 12-well plate using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Briefly, before transfection, 2x10⁵ PC-3 cells were plated in each well of a 12-well plate together with 1 ml growth medium without antibiotics and incubated until cell confluence reached 90-95%. A mix of 2 µg of plasmid and 4 µl of Lipofectamine diluted in 200 µl OptiMem (Invitrogen) was added in the confluent cells and incubated for 48 h. RKIP overexpression was verified by Western blot analysis.

Statistical analysis. Results were expressed as the mean ± SD of data obtained from independent and separate experiments. The statistical significance of differences between group means

was determined using One-way ANOVA to compared variance, using Graphpad Prism for Windows (GraphPad Software, San Diego, CA, USA). Significant differences were considered when P-value was ≤0.05.

Results

LFB-R603 is not toxic and induces cell aggregation in Ramos cells. We have examined the activity of LFB-R603 on Ramos cells and used rituximab as an internal control for comparison. Antibody concentrations ranging from 5-40 µg/ml were used. The cytotoxicity of LFB-R603 was examined following treatment of Ramos cells with various antibody concentrations and the viability was determined after 24 and 48 h of culture. Following 24-h culture, there was little toxicity observed with either antibody at any concentration used. At 48 h, there was a modest, insignificant, toxicity of LFB-R603 used at the concentration >20 µg/ml (Fig. 1A). The binding of LFB-R603 on the surface of the CD20⁺ Ramos cells was determined by flow cytometry as described in Materials and methods. There was no observed difference between the binding activity of LFB-R603 and rituximab as determined by the mean fluorescence intensity (MFI) (Fig. 1B).

Rituximab has been reported to induce cell aggregation following its interaction with various B-NHL cell lines (20,21). We investigated the ability of LFB-R603 to induce cell aggregation in Ramos cells. LFB-R603 induced cell aggregation at the concentration of 5 µg/ml and the extent of the aggregation

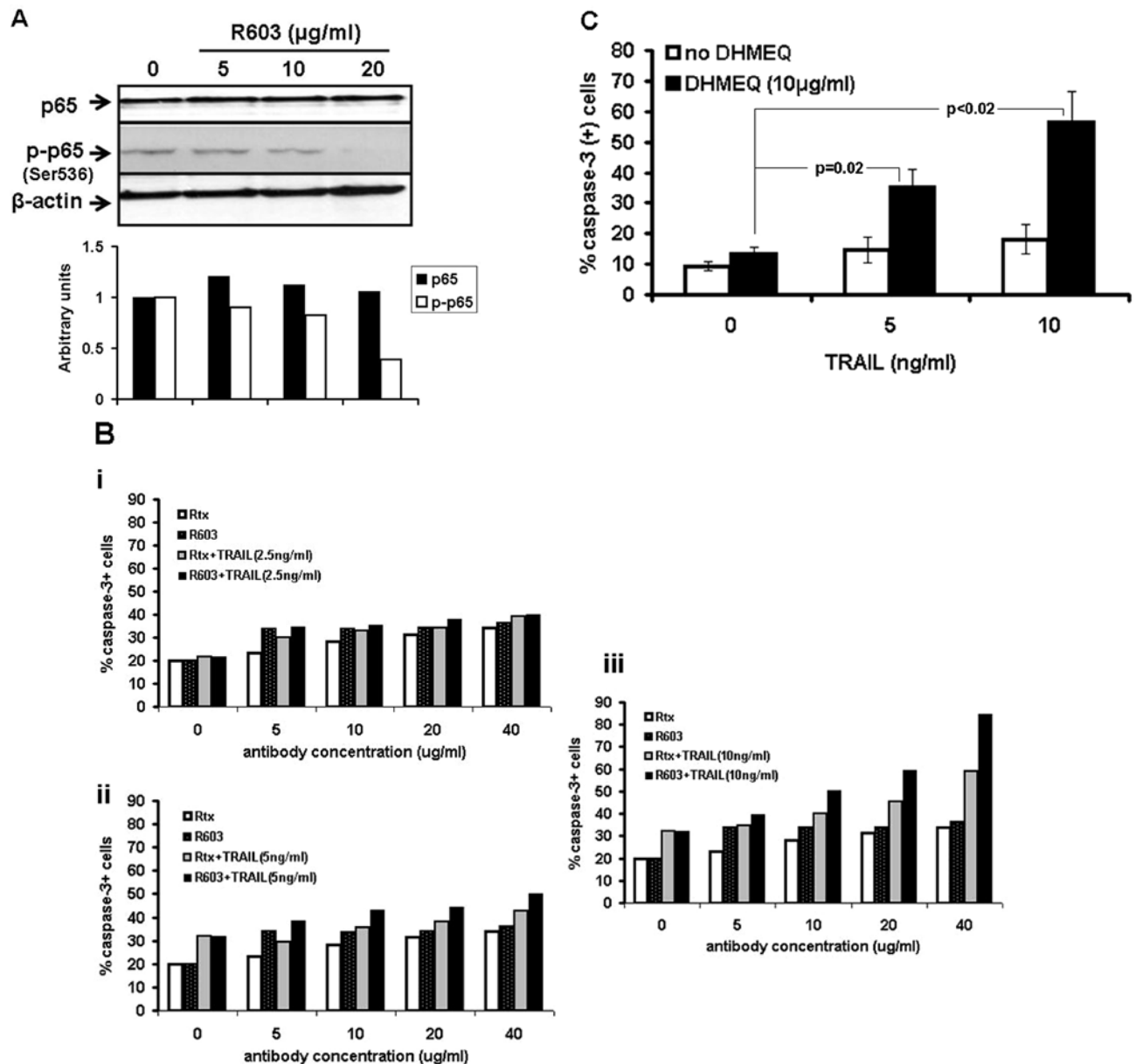


Figure 2. LFB-R603 inhibits NF- κ B activity and sensitizes Ramos cells to TRAIL-mediated apoptosis. (A) LFB-R603 inhibits NF- κ B activation. Ramos treatment with increasing concentrations of LFB-R603 for 24 h inhibits the phosphorylation of p65 NF- κ B subunit at Ser536, as assessed by Western blotting. Densitometric analysis of p65 and p-Ser536-p65 inhibition is shown in the lower panel. (B) LFB-R603 and rituximab sensitize Ramos cells to TRAIL-induced apoptosis. Cells were treated as described in Materials and methods and apoptosis was determined by flow cytometry. The percentage of apoptotic cells is expressed as percentage of caspase 3+ cells. i, TRAIL 2.5 ng/ml. ii, TRAIL 5.0 ng/ml. iii, TRAIL 10 ng/ml. (C) Direct role of NF- κ B inhibition in Ramos sensitization to TRAIL-mediated apoptosis. Ramos cells were pretreated with the indicated concentrations of the specific NF- κ B inhibitor DHMEQ for 6 h followed by treatment with 5 or 10 ng/ml TRAIL for 18 additional hours. Ramos cells were treated with various concentrations of the NF- κ B inhibitor DHMEQ and followed by treatment with TRAIL at different concentrations. Apoptosis was determined as previously described.

increased with higher antibody concentrations at both 24 and 48 h of culture (Fig. 1C).

The above findings demonstrate that LFB-R603 is not toxic to Ramos cells and induces cell aggregation at a concentration ≥ 5 μ g/ml. These findings also suggest that LFB-R603 may trigger the cells via aggregation.

LFB-R603-mediated inhibition of NF- κ B activity and sensitization of Ramos cells to TRAIL-apoptosis

1) *Inhibition of NF- κ B activity by LFB-R603.* The NF- κ B survival pathway has been reported to be constitutively activated in many solid and hematologic malignancies including NHL

(28,29). We investigated the effect of LFB-R603 on the constitutive activity of NF- κ B in Ramos cells. NF- κ B activation is regulated, in part, by phosphorylation of its subunit p65 at Ser536 (56). Thus, we analyzed by Western blotting the expression of total p65 and phospho-p65 proteins in Ramos cells treated with various concentrations of LFB-R603 for 24 h. As shown in Fig. 2A, LFB-R603 inhibited only the expression of the phosphorylated p65 protein in a concentration-dependent fashion.

2) *Sensitization of Ramos cells to TRAIL-apoptosis by LFB-R603.* Previous studies by others and ours have reported

the role of the constitutive activated NF- κ B pathway in the regulation of tumor cell response to TRAIL (57). We recently demonstrated that rituximab resensitizes TRAIL-resistant B-NHL cells to TRAIL-mediated apoptosis through inhibition of NF- κ B activity (26). Here we examined the sensitizing activity of LFB-R603 on Ramos cells for TRAIL-induced apoptosis. Ramos cells were pre-treated with various concentrations of antibody for 24 h and were followed by treatment with 2.5, 5 and 10 ng/ml of TRAIL for an additional 24 h. The cells were then harvested and examined for apoptosis detected as activation of caspase-3 as described in Materials and methods. At the TRAIL concentration of 2.5 ng/ml there was no significant apoptosis induced by either LFB-R603 or rituximab at any antibody concentration used (Fig. 2Bi). At the TRAIL concentration of 5 ng/ml, there was modest apoptosis with both LFB-R603 and rituximab, although the percent of apoptotic cells was higher when cells were treated with LFB-R603 (Fig. 2Bii). At the TRAIL concentration of 10 μ g/ml, both LFB-R603 and rituximab significantly sensitized Ramos cells to TRAIL apoptosis (Fig. 2Biii), however, LFB-R603 was more effective than rituximab. These findings demonstrate that LFB-R603 is capable of sensitizing TRAIL-resistant Ramos cells to apoptosis.

3) Direct role of LFB-R603-mediated NF- κ B inhibition in tumor sensitization to TRAIL-apoptosis. The direct role of LFB-R603-mediated inhibition of NF- κ B in tumor cell sensitization to TRAIL-apoptosis was examined by the use of the specific chemical inhibitor NF- κ B, DHMEQ. DHMEQ inhibits the nuclear translocation of NF- κ B and, therefore, its activity as transcription factor (58,59). Treatment of Ramos cells with 10 μ g/ml DHMEQ for 6 h followed by treatment with 5 or 10 ng/ml TRAIL for 18 h significantly sensitized Ramos cells to TRAIL-apoptosis. The level of apoptosis achieved was a function of the TRAIL concentration used (Fig. 2C).

The above findings demonstrate that LFB-R603 inhibits NF- κ B activity and LFB-R603-induced inhibition of NF- κ B results in the reversal of tumor cell resistance to TRAIL-apoptosis.

LFB-R603 induces RKIP expression: direct role of RKIP upregulation in Ramos cell sensitization to TRAIL-apoptosis. We have reported that RKIP, an inhibitor of NF- κ B activity, is involved in the regulation of resistance to TRAIL in prostate and melanoma cancer cell lines via different mechanisms (35,60). In addition, Jazirehi *et al* reported that induction of RKIP in B-NHL cell lines was associated with rituximab-mediated inhibition of the NF- κ B pathway; a time-dependent induction of RKIP was observed in rituximab-treated Ramos cells as early as 3-6 h post-treatment that remained at high levels up to 24 h (43). We examined whether LFB-R603-mediated NF- κ B inhibition induces RKIP expression in Ramos cells and whether the induction of RKIP plays a role in tumor sensitization to TRAIL-apoptosis. The findings derived after treatment of Ramos cells with various concentrations of rituximab or LFB-R603 for 24 h demonstrate that both antibodies induced expression of RKIP to similar extent. However, only the LFB-R603-mediated level of RKIP induction was concentration-dependent (Fig. 3A).

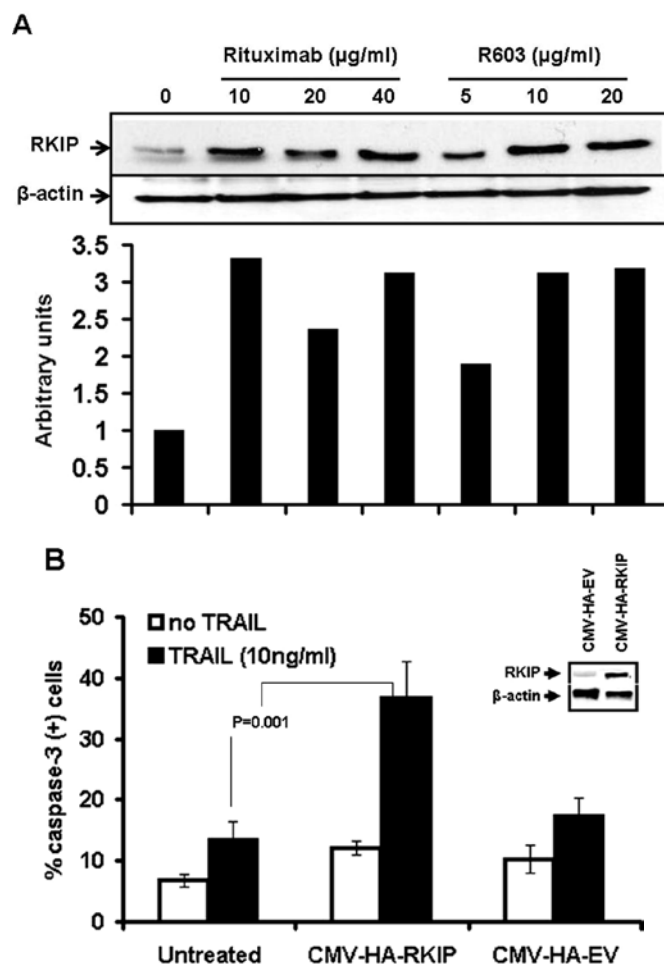


Figure 3. LFB-R603 and rituximab upregulate RKIP expression; RKIP induction sensitizes cells to TRAIL apoptosis. (A) LFB-R603- and rituximab-mediated induction of RKIP expression. Ramos cells were treated with different concentrations of LFB-R603 or rituximab for 24 h. RKIP protein expression was assessed by Western blotting as described in Materials and methods and analyzed by densitometry. β -actin was used as loading control. (B) Direct role of RKIP overexpression in cell sensitization to TRAIL apoptosis. PC-3 cells were transfected with the CMV-HA-RKIP or CMV-HA-EV vectors as described in Materials and methods. Where appropriate TRAIL was added to the cells 24 post-transfection and cells were harvested 24 h after. Apoptosis was determined by flow cytometry. RKIP overexpression was validated at 48 h post-transfection by Western blot analysis.

In order to validate the direct role of RKIP induction by LFB-R603 in tumor sensitization to TRAIL-apoptosis, PC-3 cells were transfected with the RKIP expression vector CMV-HA-RKIP, or the corresponding empty plasmid CMV-HA-EV, and, thereafter, the cells were treated with TRAIL. The findings demonstrate that only the CMV-HA-RKIP-transfected cells were able to respond to TRAIL and undergo a significant apoptosis (Fig. 3B). The transfected cells overexpressing RKIP were sensitized to TRAIL in the absence of LFB-R603. These findings demonstrate that RKIP induction has a direct role in tumor sensitization to TRAIL-apoptosis, and the LFB-R603-mediated upregulation of RKIP in Ramos cells might be critical in inducing apoptosis in response to TRAIL.

LFB-R603 inhibits Snail expression: direct role of Snail suppression in Ramos cell sensitization to TRAIL-apoptosis.

The transcription repressor and anti-apoptotic gene product Snail is, in part, under the transcriptional regulation of NF- κ B (34). We have recently reported that the inhibition of Snail in tumor cells treated with either the proteasome inhibitor NPI-0052 or the nitric oxide donor DETANONOate sensitizes tumor cells to TRAIL apoptosis (35,61,62). Therefore, we first examined whether LFB-R603-mediated inhibition of NF- κ B activity will result in the downstream inhibition of Snail expression. Ramos cells were treated with different concentrations of LFB-R603 or rituximab for 24 h and the cell lysates were examined for Snail expression by Western blotting. The data in Fig. 4A show clearly that, in contrast to rituximab, LFB-R603 was able to inhibit significantly Snail expression at concentrations of 10 and 20 μ g/ml.

The direct role of LFB-R603-mediated inhibition of Snail in Ramos sensitization to TRAIL-apoptosis was examined. Cells were transfected with Snail siRNA for 56 h and subse-

quently treated with TRAIL for another 24 h. Cells knocked down in Snail and treated with TRAIL were able to respond significantly to apoptotic signals mediated by TRAIL in contrast to those remained not transfected or transfected with the control siRNA (Fig. 4B).

The above findings demonstrate that Snail significantly confers Ramos resistance to TRAIL-apoptosis and LFB-R603-mediated Snail inhibition may be critical for cell sensitization to TRAIL.

LFB-R603 induces PTEN expression and inhibits Akt activation. Direct role of PTEN induction in Ramos sensitization to TRAIL-apoptosis. In addition to NF- κ B, the PI3K/Akt signaling cascade is one of the survival pathways which is frequently found constitutively activated in several tumors and its hyperactivation has been linked with increased cancer cell resistance to both chemotherapy and immunotherapy. PTEN functions as a negative regulator of Akt signaling via inhibition of PI3K activation and has been shown to inhibit tumor resistance when it is overexpressed. It has been recently reported that Snail negatively regulates the transcription and expression of PTEN, thus, promoting PI3K/Akt activation. Thus, we examined whether the observed inhibitory activity of LFB-R603 on Snail expression results in the induction of PTEN expression and concomitant inhibition of Akt activation. Ramos cells were treated with LFB-R603 and rituximab for 24 h and lysates were examined for the expressions of PTEN, Akt and p-Ser473-Akt by Western blotting. The data presented in Fig. 5Ai show that LFB-R603 is a more potent inducer of PTEN expression than rituximab. The induction of PTEN by both antibodies was corroborated by reduced phosphorylation of Akt in a concentration-dependent manner, thus indicating the inhibitory effect of the LFB-R603-mediated PTEN overexpression on the PI3K/Akt signaling (Fig. 5Aii). The total

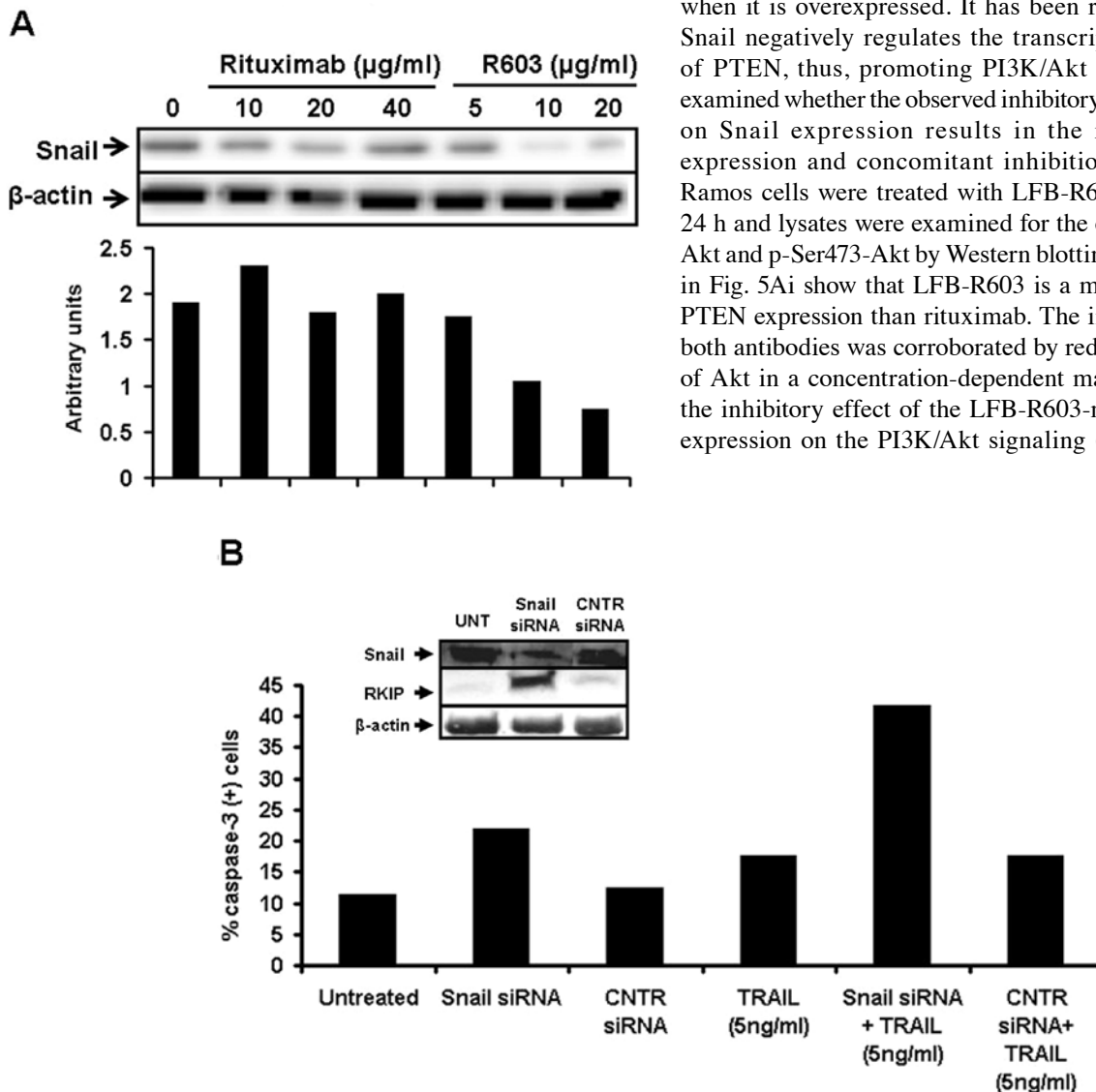


Figure 4. LFB-R603 and rituximab inhibit Snail expression; Snail suppression sensitizes cells to TRAIL apoptosis. (A) Inhibition of Snail protein levels in Ramos cells after treatment with LFB-R603 and rituximab as described above. In addition to blots, densitometric analysis of Snail expression following treatment is shown. β -actin was used as loading control. (B) Direct role of Snail inhibition in tumor sensitivity to TRAIL. Ramos cells were transfected with Snail or control siRNAs and treated with TRAIL as described in Materials and methods. Apoptosis was assessed 80 h post-transfection in transfected cells that were treated or left untreated with TRAIL. Inhibition of Snail protein levels after cell treatment with Snail siRNA was confirmed by Western blotting as shown in the upper panel.

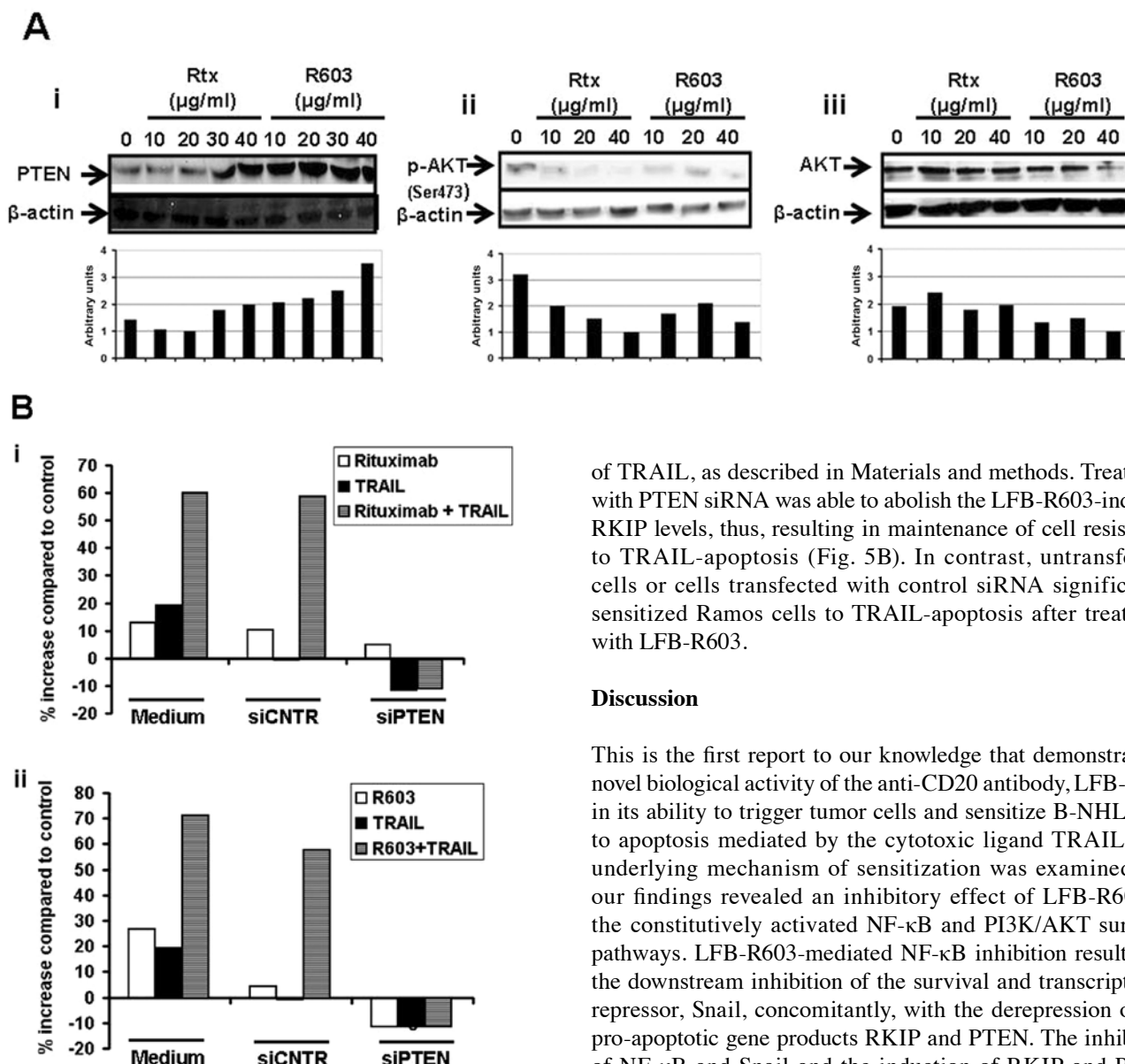


Figure 5. LFB-R603 and rituximab induce PTEN expression and suppress Akt activity. PTEN silencing reverses cell sensitization to TRAIL-apoptosis induced by LFB-R603 or rituximab. (A) LFB-R603- and rituximab-mediated induction of PTEN and suppression of Akt phosphorylation at Ser473. PTEN (i), p (Ser473)-Akt (ii) and total Akt (iii) levels were analysed in Ramos lysates treated for 24 h with the indicated concentrations of LFB-R603 and rituximab using Western blotting. β -actin was used as loading control. Densitometry for both gene products is shown in the lower panel. (B) Direct role of PTEN induction in tumor sensitization to TRAIL-apoptosis. Ramos cells were initially transfected with PTEN or control siRNAs, followed by sequential treatments with LFB-R603 (i) or rituximab (ii) and TRAIL, as described in Materials and methods. Apoptosis was determined by caspase-3 activation using flow cytometry. Graphs show the percentage of apoptosis increase after cell treatment with rituximab or LFB-R603 alone or in combination with TRAIL compared to the corresponding controls (not treated cells but transfected with either medium, CNTR siRNA, or PTEN siRNA).

Akt levels showed a slight decrease only when cells were treated with LFB-R603 (Fig. 5Aiii).

The direct role of PTEN induction by LFB-R603 in Ramos cell sensitization to TRAIL-apoptosis was examined by transfecting Ramos cells with PTEN siRNA or control siRNA and treating them with LFB-R603 in the absence or presence

of TRAIL, as described in Materials and methods. Treatment with PTEN siRNA was able to abolish the LFB-R603-induced RKIP levels, thus, resulting in maintenance of cell resistance to TRAIL-apoptosis (Fig. 5B). In contrast, untransfected cells or cells transfected with control siRNA significantly sensitized Ramos cells to TRAIL-apoptosis after treatment with LFB-R603.

Discussion

This is the first report to our knowledge that demonstrates a novel biological activity of the anti-CD20 antibody, LFB-R603 in its ability to trigger tumor cells and sensitize B-NHL cells to apoptosis mediated by the cytotoxic ligand TRAIL. The underlying mechanism of sensitization was examined and our findings revealed an inhibitory effect of LFB-R603 of the constitutively activated NF- κ B and PI3K/AKT survival pathways. LFB-R603-mediated NF- κ B inhibition resulted in the downstream inhibition of the survival and transcriptional repressor, Snail, concomitantly, with the derepression of the pro-apoptotic gene products RKIP and PTEN. The inhibition of NF- κ B and Snail and the induction of RKIP and PTEN by LFB-R603 were shown to directly regulate the tumor cell resistance to TRAIL apoptosis. Compared to rituximab, LFB-R603 showed an augmented anti-tumor activity when used at the same antibody concentrations. The findings suggest the potential therapeutic efficacy of LFB-R603 in combination with conventional cytotoxic therapies in the treatment of CD20 expressing lymphoma and leukemia cells.

Limitations of therapeutic options for B-cell malignancies such as NHL and CLL have necessitated the development of novel treatments/strategies. The introduction of therapeutic antibodies directed against the CD20 surface receptor expressed on the majority of B cells has been a milestone in the management of B-cell malignancies (17,52). Rituximab, a chimeric anti-CD20 monoclonal antibody, considerably improved the therapeutic outcomes for patients diagnosed with NHL, particularly when combined with chemotherapy; however, a significant number of patients is resistant to the initial rituximab treatment or develop a reduced response upon re-treatment (63). Although the mechanisms of resistance are not clear, it is generally believed that they are related to physical and biological properties of rituximab such as its epitope specificity, affinity, and avidity for CD20, as well as its signaling potential (16,64).

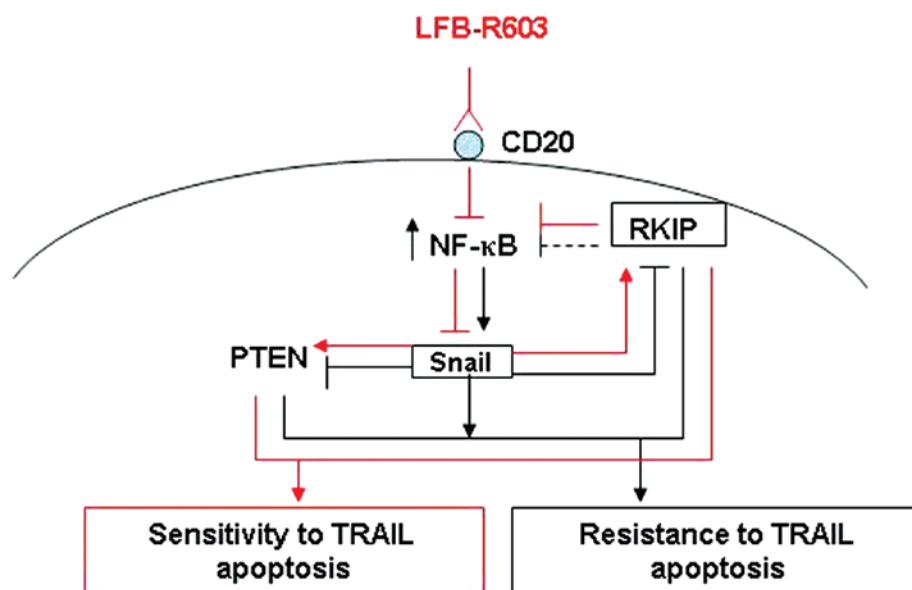


Figure 6. Schematic diagram of LFB-R603-mediated cell signaling and sensitization to TRAIL-apoptosis. This schematic diagram demonstrates that in untreated Ramos cells, the NF- κ B pathway is hyperactivated and leading downstream to expression of Snail. Snail in turn inhibits the transcription of both RKIP and PTEN. Inhibition of both RKIP and PTEN by Snail results in minimal inhibitory activity of NF- κ B (solid dark lines). Treatment of the Ramos cells with LFB-R603 results in the inhibition of NF- κ B activity and downstream inhibition of Snail expression. Inhibition of Snail results in the derepression of both RKIP and PTEN and results in their upregulation. In turn, RKIP and PTEN inhibit the NF- κ B pathway both directly and indirectly via the PI3K/Akt pathway, respectively. Altogether these gene modifications by LFB-R603 result in tumor cell sensitization to TRAIL-apoptosis (red lines).

Recent evidence suggests that the body's effector mechanisms, including complement-mediated cytotoxicity and natural killer (NK) cell-mediated killing, can be saturated or exhausted at high burdens of rituximab-opsonized B cells. One of the consequences of this saturation phenomenon is that the opsonized B cells are instead processed by a different pathway mediated by the Fc γ R on the effector cells. In this alternative pathway, both rituximab and CD20 are removed from the B cells and are taken up by monocytes/macrophages. This process of antigenic modulation may play a key role in the development of resistance to rituximab therapy (65). Therefore, the development of novel anti-CD20 mAbs with different properties than rituximab may exert a more important therapeutic activity.

Among the recently developed anti-CD20 antibodies, ofatumumab, is the most clinically advanced new anti-CD20 mAb which induces highly potent cell lysis, including cells with low CD20 expression. Positive phase III interim data for ofatumumab in fludarabine-refractory CLL that are also refractory to alemtuzumab or less suitable for alemtuzumab due to bulky (>5 cm) lymphadenopathy has led to the FDA approval of this agent in this population. Preclinical and early clinical assessment of other novel anti-CD20 mAbs include ocrelizumab, veltuzumab, GA101, AME-133v, and PRO131921; the data suggest the potential for improved efficacy over rituximab but will require substantiation in large-scale clinical trials (17). New treatment strategies and novel anti-CD20 mAbs have the potential to enhance long-term outcomes for CLL and NHL (66).

LFB-R603 is a novel chimeric mAb directed against CD20 antigen with a glycosylation profile that allows increased binding to CD16 (54). This mAb has been granted an orphan-drug status in Europe and in the USA for CLL treatment ([http://](http://www.lfb.fr/en/home.html)

www.lfb.fr/en/home.html). A previous generation of this antibody, named EMAB-6, sharing the same specificity and a similar glycosylation pattern, was described by De Romeuf *et al* (55). The FccRIIIA-optimized LFB-R603 mAb has been shown to exhibit 100 times greater antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells from CLL patients and healthy donors than rituximab. In the presence of NK cells from CLL patients LFB-R603 could also kill the same number of Raji cells (B-NHL) at 1/100 the concentration of rituximab (54). To our knowledge, except ADCC, no other cytotoxic mechanism activated by LFB-R603 has been described so far for both CLL and NHL. In addition, there are no available reports on LFB-R603 intracellular signaling modulation related to cell survival.

Rituximab has been reported to signal NHL cells and inhibit intracellular survival anti-apoptotic signaling pathways leading to inhibition of proliferation and cell sensitization to various drugs and cytotoxic ligands (22,67-69). Hence, the present study investigated the ability of LFB-R603 to i) signal NHL cells ii) inhibit the constitutively activated survival pathway NF- κ B and consequently to iii) regulate tumor cell resistance to apoptotic stimuli such as TRAIL. Treatment of Ramos cells with LFB-R603 had no direct toxicity and resulted in significant tumor cell aggregation dependent on the concentration of LFB-R603 used. These findings suggest that LFB-R603 may be signaling the cells via interaction with the CD20 receptor. Our data demonstrate that treatment of TRAIL-resistant Ramos cells with LFB-R603 significantly inhibited NF- κ B activity and cell resistance to TRAIL-mediated apoptosis was reversed. The direct role of NF- κ B-mediated inhibition by LFB-R603 in the sensitization was corroborated by findings demonstrating that treatment of Ramos cells with the specific NF- κ B inhibitor, DHMEQ, sensitized the cells to

TRAIL-apoptosis and, thus, mimicking LFB-R603. The mechanism by which the inhibition of NF- κ B activity resulted in sensitization was further explored by examining the role of the resistance-related NF- κ B target, Snail. The inhibition of Snail correlated with the inhibition of NF- κ B by LFB-R603. The direct role of Snail inhibition of LFB-R603 in the sensitization to TRAIL was demonstrated in tumor cells transfected with Snail siRNA and such cells reversed their resistance to TRAIL-apoptosis.

Since Snail is a transcription repressor of both RKIP and PTEN, we further investigated whether LFB-R603-mediated Snail inhibition resulted in derepression of RKIP and PTEN and induction of their expression. Indeed, the findings demonstrate that following treatment of Ramos cells with LFB-R603 both RKIP and PTEN were significantly induced. The role of RKIP induction by LFB-R603 in tumor sensitization to TRAIL was shown by RKIP overexpression. Cells overexpressing RKIP were resensitized to TRAIL apoptosis, thus, indicating the direct role of RKIP in the reversal of tumor resistance to TRAIL. Likewise, LFB-R603 induced the expression of PTEN and resulting in inhibition of PI3/Akt activation. Cell treatment with PTEN siRNA abolished the LFB-R603-mediated Ramos sensitivity to TRAIL-apoptosis, and, therefore, suggesting that PTEN has a direct role in the sensitizing mechanism of LFB-R603. Overall, these findings demonstrate that LFB-R603 resensitizes resistant Ramos cells to TRAIL-apoptosis through modification of the dysregulated NF- κ B/Snail/RKIP/PTEN circuitry. In this loop, both NF- κ B and Snail regulate resistance whereas both RKIP and PTEN regulate sensitivity. These findings extend our knowledge on the cytotoxic mechanisms of LFB-R603 suggesting that besides the direct activation of ADCC and CDC, LFB-R603 can indirectly kill B-cell malignant cells by sensitizing them to apoptosis mediated by various chemotherapeutic drugs or cytotoxic ligands.

The findings presented in this study with LFB-R603 were compared to the activity mediated by rituximab. In several instances, the activity of LFB-R603 was superior to that mediated by rituximab based on the same concentration of antibody used. There were primarily quantitative differences but not necessarily qualitative differences observed. We speculate that these differences could be mainly attributed to diverse activating signaling potential and efficiency triggered by the two antibodies. Our findings further suggest that the combination treatment of LFB-R603 and rituximab may result in an additive effect. Such studies are currently being examined. In addition, the combination of LFB-R603 with other anti-CD20 antibodies may result in additive or synergistic effects. Collectively, our findings suggest that the unbalanced NF- κ B/Snail/RKIP/PTEN circuitry in Ramos cells has a critical impact in the regulation of tumor cells response to TRAIL and most likely to other apoptotic stimuli. The interregulatory activity of each of the components of this circuitry is schematically illustrated in Fig. 6. We established that LFB-R603 inhibits NF- κ B and Snail and induces RKIP and PTEN and each of these gene products is involved in the regulation of tumor cells sensitivity to TRAIL. However, we do not rule out that all or some of these gene products in the loop also may regulate tumor cell resistance or sensitivity by distinct mechanisms not involving the loop. The NF- κ B/Snail/RKIP/PTEN feedback loop is dysregulated in cancer cells with

an overall effect in cell survival and resistance. Each gene product may also have a prognostic significance. The findings also support that LFB-R603 is of therapeutic significance when used in combination with conventional therapeutics. Since TRAIL is currently in clinical trials, we suggest that treatment with LFB-R603 in combination with TRAIL may potentiate the efficacy of the treatment. Likewise, since TRAIL is expressed on the surface of immune cells such as NK and lymphocytes, treatment with LFB-R603 may sensitize the tumor cells to host immune effector cells expressing TRAIL and other ligands of the TNF- α family. Based on the more potent activity of LFB-R603 compared to rituximab in some of our experimental approaches, we also suggest that it may have a more augmented therapeutic capacity than rituximab. Further investigation of intracellular signaling triggered by LFB-R603 as well as *in vivo* validation of its sensitizing activity to apoptosis induced by chemotherapy or other immunotherapy in models of B-cell malignancies resistant to current immunotherapeutic approaches can potentially add to the better understanding of the anti-tumor activities of this novel anti-CD20 mAb.

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