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## Dimeric procyanidin B2 inhibits constitutively active NF- $\kappa$ B in Hodgkin's lymphoma cells independently of the presence of I $\kappa$ B mutations

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### ABSTRACT

Due to long-term toxicity of current Hodgkin's lymphoma (HL) treatment, the present challenge is to find new therapies that specifically target deregulated signaling cascades, including NF- $\kappa$ B, which are involved in Hodgkin (H) and Reed-Sternberg (RS) cell proliferation and resistance to apoptosis. We previously presented evidence that dimeric procyanidin B2 (B2) can interact with NF- $\kappa$ B proteins inhibiting the binding of NF- $\kappa$ B to DNA. Herein, we investigated if B2, acting at a late event in NF- $\kappa$ B signaling cascade, could be effective in inhibiting NF- $\kappa$ B in H-RS cells with different mechanisms of constitutive NF- $\kappa$ B activation. B2 caused a concentration-dependent inhibition of NF- $\kappa$ B-DNA binding to a similar extent (41–48% inhibition at 25  $\mu$ M B2) in all the tested H-RS cell lines (L-428, KM-H2, L-540, L-1236 and HDML-2). This was associated with the inhibition of NF- $\kappa$ B-driven gene expression, including cytokines (IL-6, TNF $\alpha$  and RANTES) and anti-apoptotic proteins (Bcl-xL, Bcl-2, XIAP and cFLIP). The finding of similar amounts of RelA and p50 proteins in the nucleus, but decreased NF- $\kappa$ B-DNA binding, even in those H-RS cells characterized by mutations in the inhibitory I $\kappa$ B proteins, supports that B2 acts by preventing the binding of NF- $\kappa$ B to DNA. B2 did not inhibit AP-1 and STAT3 constitutive activation in H-RS cells, indicating that the moderate effects of B2 on cell viability are due to the complex signaling aberrations in HL. Thus, several signaling pathways should be targeted when designing therapeutics for HL. In this regard, the capacity of B2 to inhibit NF- $\kappa$ B could be valuable in a multi-drug approach.

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**Abbreviations:** DCDHF, 5(or 6)-carboxy-2'-7'-dichlorodihydrofluorescein diacetate; DCF, 2'-7'-dichlorofluorescein; DTT, dithiothreitol; EBV, Epstein-Barr virus; EDTA, ethylenediamine tetraacetate; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HL, Hodgkin's lymphoma; H-RS cells, Hodgkin and Reed-Sternberg cells; IKK, I $\kappa$ B kinase; IL-6, interleukin-6; LMP-1, latent membrane protein-1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PBS, phosphate-buffered saline; RANTES, Regulated on Activation Normal T cell Expressed and Secreted; TNF $\alpha$ , tumor necrosis factor alpha.

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## 1. Introduction

Treatment of Hodgkin's lymphoma (HL) in the last decades with chemotherapy and a multi-drug approach has been successful. However, 20–30 years after the treatment, its toxicity becomes evident as secondary malignancies and cardiovascular disease (reviewed in [1]). Therefore, the current challenge in HL treatment is to find new therapies that specifically target deregulated signaling cascades that cause H-RS cell proliferation and resistance of apoptosis. HL is morphologically characterized by the presence of Hodgkin (H) and Reed-Sternberg (RS) cells that generally constitute less than 1% of the total tumor cell mass. The malignant H-RS cells are surrounded by a stromal background of hyperplastic cells, including reactive non-neoplastic lymphocytes, histiocytes, plasma cells, eosinophils and stromal cells. These reactive cells are attracted by an array of cytokines and chemokines produced in large amounts by H-RS cells [2–4]. Several of these cytokines and chemokines are regulated by transcription factor NF- $\kappa$ B. In fact, H-RS cells are characterized by a constitutive activation of NF- $\kappa$ B that could explain the transformation of germinal B cells into H-RS cells and also elucidate the clinical and pathological characteristics of HL [5]. In this regard, NF- $\kappa$ B pathway emerges as one key signaling cascade to be targeted in the treatment of HL.

NF- $\kappa$ B is formed by homo and heterodimers of the Rel/NF- $\kappa$ B family of proteins. It is sequestered in the cytosol in an inactive form by the interaction with members of the inhibitory I $\kappa$ B proteins [6]. For activation of the classical pathway, two conserved serines in I $\kappa$ B are phosphorylated by specific I $\kappa$ B kinases (IKK), which target I $\kappa$ B for ubiquitination and degradation by the proteasome [7]. Degradation of I $\kappa$ B unmasks the nuclear localization signal allowing the nuclear translocation of NF- $\kappa$ B, binding to its consensus sequence and the transactivation of NF- $\kappa$ B-dependent genes [8]. Given its capacity to modulate the expression of genes involved in inflammation, cell proliferation and survival, together with the finding of its constitutive activation in many different cancer types, suggest that NF- $\kappa$ B has an active role in carcinogenesis [9,10]. Indeed, the activation of NF- $\kappa$ B has been described in solid tumors (breast, gastric and colonic cancers) as well as in leukemia and lymphoma (reviewed in [11]), including HL [12,13].

Procyanidins are oligomers of flavan-3-ol subunits present in large amount in certain edible plants and plant-derived foods (cocoa, grapes, cranberries, apples, red wine). Dimeric procyanidin B2 is composed of 2 molecules of the flavan-3-ol (–)-epicatechin linked by a 4 $\beta$  → 8 bonds (Fig. 1). B2 can inhibit transcription factor NF- $\kappa$ B at different levels in this signaling pathway. However, the most important characteristic is that B2 exerts a selective inhibition of NF- $\kappa$ B in the nucleus by preventing the binding of the active NF- $\kappa$ B to DNA [14]. The molecular modeling of the minimum energy conformer of B2 resulted in a folded structure where the hydroxyl groups of rings B' and A orient in the same direction [14]. We showed that these hydroxyl groups could establish hydrogen bonds with the NF- $\kappa$ B proteins RelA and p50, thus inhibiting the binding of NF- $\kappa$ B to DNA  $\kappa$ B sites.

Given the capacity of B2 to inhibit a late event in the NF- $\kappa$ B signaling cascade [14], we hypothesize that B2 could be

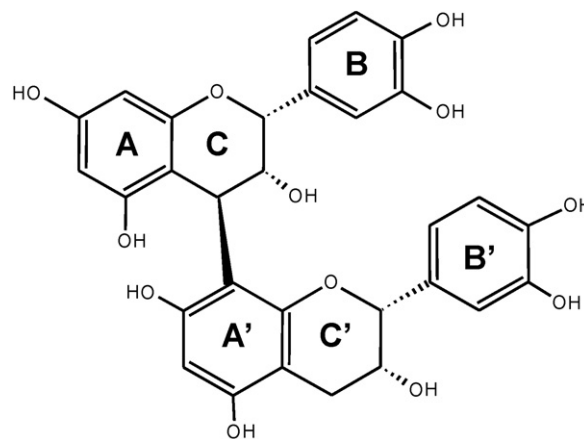


Fig. 1 – Chemical structure of procyanidin B2.

effective in the inhibition of NF- $\kappa$ B in H-RS cells, even when constitutive NF- $\kappa$ B activation is secondary to mutations in the inhibitory I $\kappa$ B proteins [15–18]. For this purpose, we investigated the capacity of B2 to inhibit NF- $\kappa$ B in H-RS cell lines with different underlying causes of constitutive activation. Furthermore, the potential modulation of B2 on constitutive AP-1 and STAT3 activation was assessed. Based on extensive experimental evidence supporting their correspondence to HL malignant cells [1], H-RS cell lines were used as the experimental model. Our data indicate that B2 can inhibit constitutive NF- $\kappa$ B activation in H-RS cells by preventing the binding of NF- $\kappa$ B to its consensus sequence. In addition, the capacity of B2 to inhibit NF- $\kappa$ B-regulated anti-apoptotic proteins could be of potential use to decrease the resistance of H-RS cells to undergo apoptosis and therefore make them more susceptible to the action of other chemotherapeutic agents.

## 2. Materials and methods

### 2.1. Materials

Procyanidin B2 [epicatechin-(4 $\beta$  → 8)-epicatechin] was obtained from ExtraSynthese (Genay, France). The H-RS cells (KM-H2, L-428, L-540, L-1236 and HDLM-2) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), while Jurkat (T cell leukemia, human) and Daudi (B lymphoblast originated from Burkitt's lymphoma, human) cells were obtained from the American Type Culture Collection (Rockville, MA). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). The oligonucleotides containing the consensus sequence for NF- $\kappa$ B, AP-1 and OCT-1 as well as the reagents for the electrophoretic mobility shift assay (EMSA) were obtained from Promega (Madison, WI). Tumor necrosis factor (TNF $\alpha$ ) ELISA kit was obtained from BD Biosciences (San Diego, CA). Regulated on Activation Normal T cell Expressed and Secreted (RANTES) ELISA kit was obtained from Biosource (Camarillo, CA). The protease inhibitor cocktail and the interleukin-6 (IL-6) ELISA kit were obtained from Roche Applied Science (Man-

nheim, Germany). The antibodies for cFLIP, Bcl-xL, I $\kappa$ B $\alpha$ , RelA, p50 and  $\beta$ -tubulin, and the oligonucleotide containing the consensus sequence for STAT3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-2, XIAP, phospho-STAT3 (tyr 705), STAT3 and phospho-I $\kappa$ B $\alpha$  (ser 32) antibodies were obtained from Cell Signaling Technology (Beverly, MA). PVDF membranes were obtained from BIO-RAD (Hercules, CA) and Chroma Spin-10 columns were obtained from Clontech (Palo Alto, CA). The ECL Western blotting system was from GE Healthcare (formerly Amersham Pharmacia Biotech. Inc.) (Piscataway, NJ). Propidium iodide and 5(or 6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate (DCDHF) were obtained from Molecular Probes (Eugene, OR). All other reagents were from the highest quality available and were purchased from Sigma (St. Louis, MO).

## 2.2. Cell culture and incubations

H-RS, Daudi and Jurkat cells were cultured in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum and antibiotics (10 U/ml penicillin and 10  $\mu$ g/ml streptomycin). Cells were incubated with B2 (2.5–100  $\mu$ M) for variable periods of time (3–72 h). The number of viable cells was measured evaluating the cell ATP content using the CellTiter-Glo Luminiscent Cell Viability assay (Promega, Madison, WI).

## 2.3. Determination of cell oxidants

Cell oxidants were evaluated as previously described in Ref. [14] using the probe DCDHF. This probe can cross the membrane and fluoresces when it is oxidized inside the cell. Cells ( $1 \times 10^5$ ) were incubated in the absence or presence of B2 for 3–48 h. Subsequently, cells were centrifuged at  $800 \times g$  for 10 min, rinsed with warm PBS and suspended in 200  $\mu$ l of RPMI 1640 medium containing 10  $\mu$ M DCDHF. After 30 min of incubation at 37 °C, the media were removed; cells were rinsed with PBS, and then incubated in 200  $\mu$ l of PBS containing 0.1% (v/v) Igepal. After a brief sonication and 30 min incubation with regular shaking, the fluorescence at 525 nm ( $\lambda_{exc}$ : 475 nm) was measured. To evaluate DNA content, samples were added with 50  $\mu$ M propidium iodide. After 20 min of incubation at room temperature, the fluorescence ( $\lambda_{exc}$ : 538,  $\lambda_{em}$ : 590) was measured. Results are expressed as the ratio DCF fluorescence/propidium iodide fluorescence.

## 2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear fractions were isolated as previously described in Ref. [14]. After the corresponding treatment, cells ( $2 \times 10^6$  cells) were collected by centrifugation at  $800 \times g$  for 10 min. Cells were resuspended in 150  $\mu$ l of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1% (v/v) Igepal), incubated for 10 min at 4 °C and then centrifuged for 1 min at  $12,000 \times g$ . The supernatant fraction was discarded and the nuclear pellets resuspended in 60  $\mu$ l of buffer B (10 mM Hepes, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF). Samples were incubated for 20 min at 4 °C and centrifuged at  $10,000 \times g$  for 15 min at 4 °C. The supernatant was transferred to a new tube,

protein concentration was determined by the method of Bradford [19] and samples were stored at –80 °C.

For the EMSA, the oligonucleotides containing the consensus sequences for NF- $\kappa$ B, AP-1, OCT-1 and STAT3 were end labeled with [ $\gamma$ -<sup>32</sup>P] ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1 $\times$  binding buffer (5 $\times$  10 mM Tris-HCl buffer, pH 7.5, containing 4% (v/v) glycerol, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl and 0.05 mg/ml poly(dI-dC)). The products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrylamide gel using 0.5 $\times$  TBE (Tris/borate 45 mM, EDTA 1mM) as the running buffer. The gels were dried and the radioactivity was quantitated in a Phosphoimager 840 (GE Healthcare, Piscataway, NJ).

## 2.5. Western blot analysis

Nuclear fractions were obtained as described above and total cell fractions as previously described in Ref. [14]. Aliquots of nuclear or total fractions containing 25–40  $\mu$ g protein were separated by reducing 10–12.5% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Rainbow molecular weight standards (GE Healthcare, Piscataway, NJ) were run simultaneously. For RelA and p50, membranes were blocked overnight in 5% (w/v) non-fat milk and subsequently incubated in the presence of the corresponding antibodies (1:1000 dilution) for 90 min at 37 °C. After incubation for 90 min at room temperature in the presence of the secondary antibody (HRP-conjugated) (1:5000 dilution), the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840. To evaluate the bcl-2, bcl-xL, XIAP, cFLIP, phospho-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , phospho-STAT3 and STAT3 proteins, membranes were immunoblotted with the corresponding primary antibody (1:500 dilution) in 5% (w/v) bovine serum albumin overnight at 4 °C and the following day for 90 min at room temperature in the presence of the corresponding secondary antibody (HRP-conjugated). The conjugates were detected by enhanced chemiluminescence in a Phosphoimager 840 (GE Healthcare, Piscataway, NJ).

## 2.6. Measurement of cytokines

Cytokines were measured by ELISA. Briefly, cells ( $1.5 \times 10^6$ ) were incubated for 48 h in the different experimental conditions. Cytokines (IL-6, TNF $\alpha$  and RANTES) released to the media were measured after separating the cells by centrifugation at  $800 \times g$  for 10 min. IL-6, TNF $\alpha$  and RANTES were measured by ELISA assays following the manufacturer's protocols.

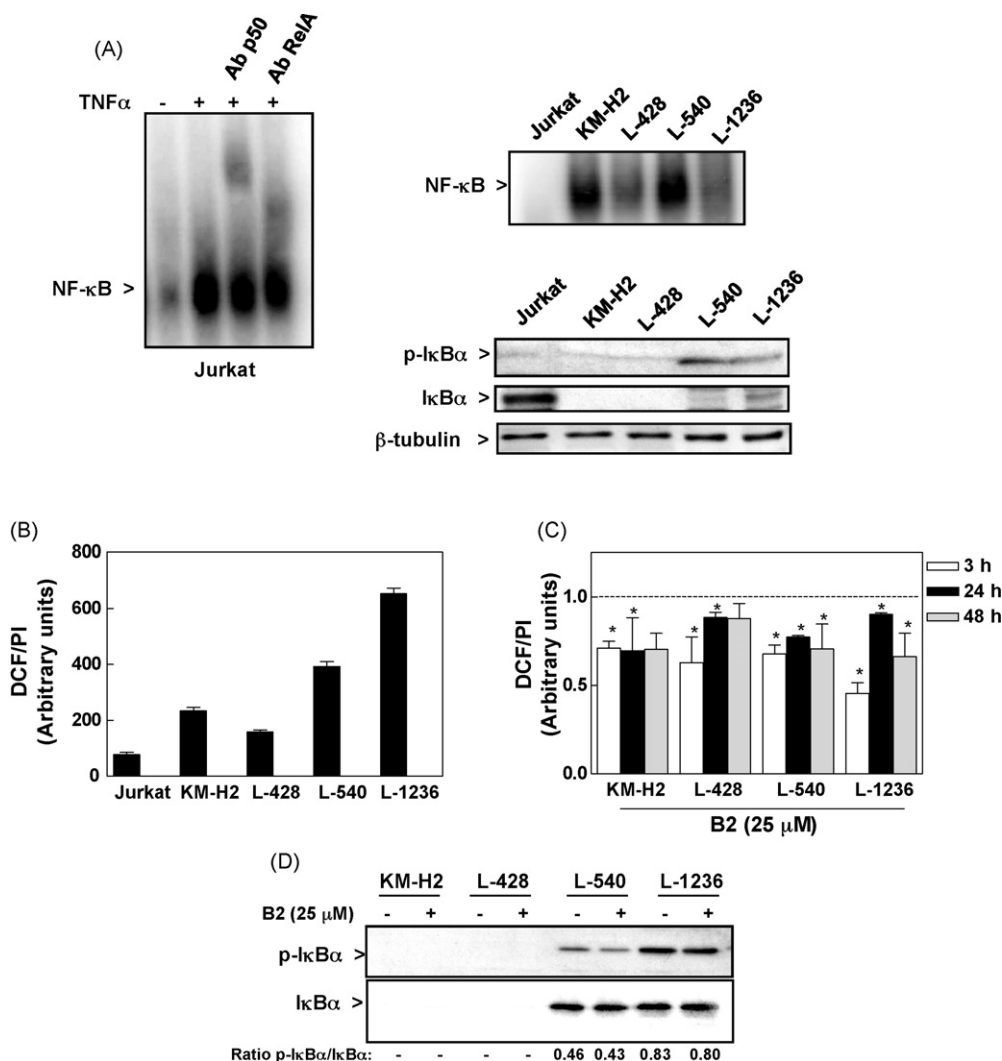
## 2.7. Statistical analysis

One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffe was performed using Statview 512+ (Brainpower Inc., Calabasas CA). A *p* value < 0.05 was considered statistically significant. Values are given as mean  $\pm$  S.E.M.

### 3. Results

Hodgkin's cells consistently show a constitutive activation of NF- $\kappa$ B (Fig. 2A) as a consequence of different mechanisms of aberrant NF- $\kappa$ B activation. We have previously presented evidence that B2 could interact with NF- $\kappa$ B proteins inhibiting the binding of NF- $\kappa$ B to the DNA [14]. Thus, because B2 inhibits a late event in the NF- $\kappa$ B signaling cascade, we hypothesize that it could be effective in the

inhibition of NF- $\kappa$ B in H-RS cells regardless of the presence of functional I $\kappa$ B inhibitory proteins. Low levels of I $\kappa$ B $\alpha$  were observed in L-540 and L-1236 cells compared to Jurkat T cells (Fig. 2A). These occur secondary to the activation of upstream events of the NF- $\kappa$ B pathway, which are still not fully characterized. KM-H2 and L-428 cells are characterized by mutations that lead to the absence of functional I $\kappa$ B $\alpha$  or of I $\kappa$ B  $\alpha$  and I $\kappa$ B $\epsilon$ , respectively (Fig. 2A) [5,16,17].



**Fig. 2 – Constitutive NF- $\kappa$ B activation and cell oxidant levels in H-RS cells. (A)** Upper left panel: NF- $\kappa$ B-DNA binding activity in nuclear fractions isolated from Jurkat cells incubated in the absence or presence of 20 ng/ml TNF $\alpha$  for 30 min. To determine the protein components of the active NF- $\kappa$ B, TNF $\alpha$ -treated samples were incubated in the presence of antibodies for p50 or RelA, for 30 min prior to the binding assay. Upper right panel: NF- $\kappa$ B-DNA binding activity in nuclear fractions isolated from Jurkat, KM-H2, L-428, L-540 and L-1236 cells measured by EMSA. Lower panel: phosphorylated I $\kappa$ B $\alpha$  (p-I $\kappa$ B $\alpha$ ) and I $\kappa$ B $\alpha$  levels in total fractions isolated from Jurkat, KM-H2, L-428, L-540 and L-1236 cells measured by Western blot.  $\beta$ -tubulin is shown as loading control. **(B)** Basal oxidant levels were evaluated with the probe DCFH<sub>2</sub> as described under Section 2. DCF fluorescence was normalized to the propidium iodide (PI) fluorescence. **(C)** Cells were incubated for 3–48 h without or with 25  $\mu$ M B2. DCF fluorescence was normalized to the propidium iodide (PI) fluorescence and the values are referred to those obtained for cells incubated in the absence of B2 (1.0). **(B and C)** Results are shown as mean  $\pm$  S.E.M. of 3 independent experiments. (\*) Significantly different from the untreated group ( $p < 0.05$ , one-way ANOVA test). **(D)** Phosphorylated I $\kappa$ B $\alpha$  (p-I $\kappa$ B $\alpha$ ) and I $\kappa$ B $\alpha$  levels were measured by Western blot in total cell fractions isolated from L-428, KM-H2, L-540 and L-1236 cells incubated for 24 h in the absence or presence of 25  $\mu$ M B2. One representative experiment is shown.

3.1. H-RS cells are characterized by a high production of cell oxidants

We previously observed that KM-H2 and L-428 cells are characterized by a high production of cell oxidants [20]. Therefore, we investigated the relative levels of cell oxidants in the different H-RS cell lines and its relationship to the constitutive levels of NF-κB activation. The levels of cell oxidants were measured with the probe DCDHF and results were referred to the DNA content (measured with propidium iodide). When compared to Jurkat T cells as a model of T cells

without a constitutive activation of NF-κB, cell oxidant levels were 3-, 2-, 5- and 8.3-fold higher in KM-H2, L-428, L-540 and L-1236, respectively (Fig. 2B). The incubation of cells in the presence of 25 μM B2 for 3–48 h caused a partial decrease of cell oxidant levels (29–55% decrease, after 3 h of incubation) (Fig. 2C). The constitutive levels of NF-κB activation measured by EMSA in nuclear extracts did not show a correlation with levels of cell oxidants (Fig. 2A and B).

Next we evaluated if the increase in cell oxidant levels could lead to the activation of upstream events in the NF-κB pathway. After 24 h of incubation, no significant

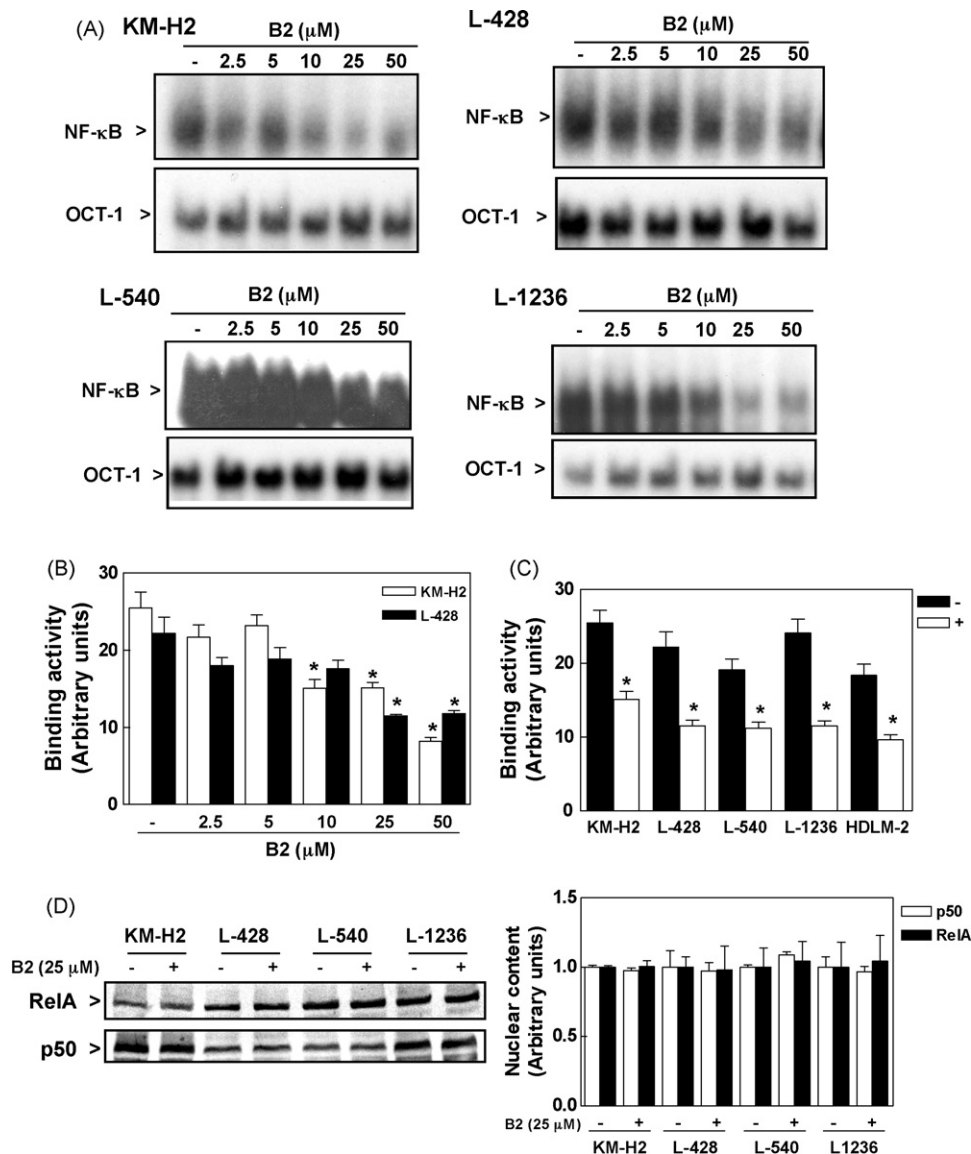


Fig. 3 – B2 inhibits NF-κB-DNA binding in H-RS cells. NF-κB-DNA binding was measured by EMSA in nuclear fractions isolated from L-428, KM-H2, L-540, L-1236 and HDLM-2 cells incubated for 24 h in the absence or presence 2.5–50 μM B2. (A) One representative experiment is shown. (B–D) After the corresponding assays, bands were quantitated and results are shown as mean ± S.E.M. of 3 independent experiments. (\*) Significantly different compared to untreated cells ( $p < 0.05$ , one-way ANOVA test). (B) B2 dose (2.5–50 μM)-dependent inhibition of NF-κB-DNA binding in KM-H2 (empty bars) and L-428 (full bars). (C) Inhibitory action of B2 on NF-κB-DNA binding in KM-H2, L-428, L-540, L-1236 and HDLM-2 incubated for 24 h in the absence (full bars) or presence (empty bars) of 25 μM B2. (D) RelA and p50 content was measured by Western blot in nuclear fractions isolated from cells incubated for 24 h without (–) or with (+) 25 μM B2.

differences in I $\kappa$ B $\alpha$  phosphorylation and I $\kappa$ B $\alpha$  total levels were observed in L-540 and L-1236 cells incubated without or with 25  $\mu$ M B2 (Fig. 2D). No detectable levels of phosphorylated I $\kappa$ B $\alpha$  and I $\kappa$ B $\alpha$  were observed in KM-H2 and L428 cells (Fig. 2D).

3.2. B2 inhibits NF- $\kappa$ B-DNA binding in H-RS cells with different mechanisms of constitutive NF- $\kappa$ B activation

The incubation of KM-H2, L-428, L-540, L-1236 and HDLM-2 cells for 24 h in the presence of B2 led to a dose (2.5–50  $\mu$ M)-

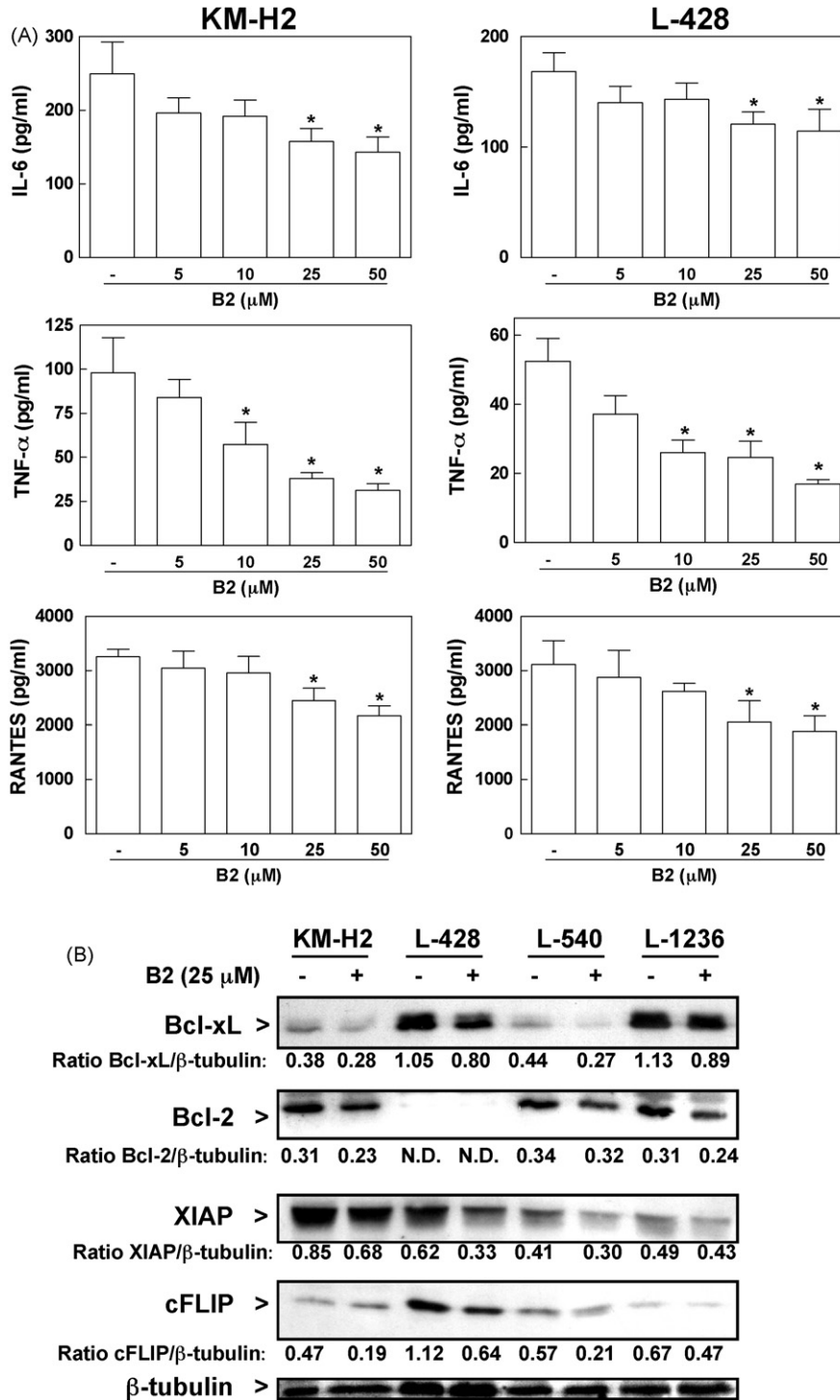


Fig. 4 – B2 inhibits NF- $\kappa$ B-regulated gene expression in H-RS cells. (A) KM-H2 and L-428 cells were incubated for 48 h in the absence or presence of 2.5–50  $\mu$ M B2. The concentration of IL-6, TNF $\alpha$  and RANTES released to the media was measured by ELISA. Results are mean  $\pm$  S.E.M. of 3 independent experiments. (\*) Significantly different compared to untreated cells ( $p < 0.05$ , one-way ANOVA test). (B) KM-H2, L-428, L-540 and L-1236 cells were incubated for 48 h in the absence or presence of 25  $\mu$ M B2. The content of different anti-apoptotic proteins was evaluated by Western blot in total cell fractions as described in Section 2. One representative experiment out of 3 independent experiments is shown. N.D.: non-detectable.

dependent inhibition of NF- $\kappa$ B-DNA binding in nuclear fractions as measured by EMSA (Fig. 3A and B). The lowest concentration that caused a significant inhibitory effect was 10  $\mu$ M B2 in KM-H2 cells. The extent of inhibition (41–48%) of NF- $\kappa$ B-DNA binding at 25  $\mu$ M B2 was similar for the five cell lines investigated (Fig. 3C). These results indicate that B2 can inhibit NF- $\kappa$ B-DNA binding in all H-RS cells investigated, regardless of the absence of functional I $\kappa$ B inhibitory proteins.

The nuclear content of the main NF- $\kappa$ B protein components RelA and p50 was similar in H-RS cells incubated for 24 h in the absence or presence of 25  $\mu$ M B2 (Fig. 3D). Given that under the same experimental conditions NF- $\kappa$ B-DNA nuclear binding was significantly lower in the H-RS cells treated with 25  $\mu$ M B2 (Fig. 3C), these results support our proposed interaction of B2 with NF- $\kappa$ B proteins and therefore preventing the binding of the active NF- $\kappa$ B to its DNA  $\kappa$ B sites.

We subsequently studied the consequences of the inhibitory action of B2 on NF- $\kappa$ B-DNA binding on the NF- $\kappa$ B-dependent transactivation of genes associated with the ethiological, clinical and pathological characteristics of HL.

### 3.3. B2 inhibits the release of NF- $\kappa$ B-dependent cytokines in H-RS cells

H-RS cells produce an array of cytokines and chemokines that attract a number of reactive cells (non-neoplastic lymphocytes, histiocytes, plasma cells, eosinophils and stromal cells) [2–4]. Several of these cytokines are regulated by NF- $\kappa$ B. Thus, we investigated if the B2-mediated inhibition of NF- $\kappa$ B-DNA binding could lead to a decreased transactivation of NF- $\kappa$ B-dependent cytokines (IL-6, TNF $\alpha$  and RANTES).

KM-H2, L-428, L-540, L-1236 and HDLM-2 cells were incubated for 48 h in the presence of B2 and the concentration of IL-6, TNF $\alpha$  and RANTES released was measured in the media by ELISA. Treatment of KM-H2 and L-428 cells with B2 led to a dose (2.5–50  $\mu$ M)-dependent inhibition of IL-6, TNF $\alpha$  and RANTES secretion (Fig. 4A). The effect was significant at concentrations of 25  $\mu$ M or higher for IL-6 and RANTES and at 10  $\mu$ M or higher for TNF $\alpha$  in KM-H2 and L-428 cells. The amount of IL-6, TNF $\alpha$  and RANTES released to the media after 48 h shows a large variation among the different H-RS cells (Table 1). The largest inhibitory effects of B2 were observed for

TNF $\alpha$  (33–87%). The inhibition of IL-6 secretion by B2 ranged from 17% to 58% among the different cell lines and for RANTES was similar for all the cell lines (25–38%) (Table 1). Thus, the inhibitory effect of B2 depended on the cytokine and on the cell line. In this regard, the effect of B2 depended in general on the basal level of cytokine secretion by each particular H-RS cell line.

### 3.4. B2 inhibits the expression of NF- $\kappa$ B-dependent anti-apoptotic proteins

Given the role of NF- $\kappa$ B in the resistance of cells to die by apoptosis, we subsequently investigated the effects of B2 on the expression of NF- $\kappa$ B-regulated proteins involved in the prevention of apoptosis (Bcl-xL, Bcl-2, XIAP, cFLIP).

As depicted in Fig. 4B, the studied H-RS cells were characterized by a remarkably different pattern of expression of anti-apoptotic proteins. The incubation of H-RS cells for 48 h in the presence of 25  $\mu$ M B2 caused a decreased content of the Bcl-xL, Bcl-2, XIAP and cFLIP as measured by Western blot in total cell fractions (Fig. 4B). However, the incubation of cells for 24–72 h in the presence of 25  $\mu$ M B2 did not cause a significant induction of apoptotic cell death as evaluated by the cell binding of Annexin-V, which is associated to phosphatidylserine externalization as an early marker of apoptosis (data not shown).

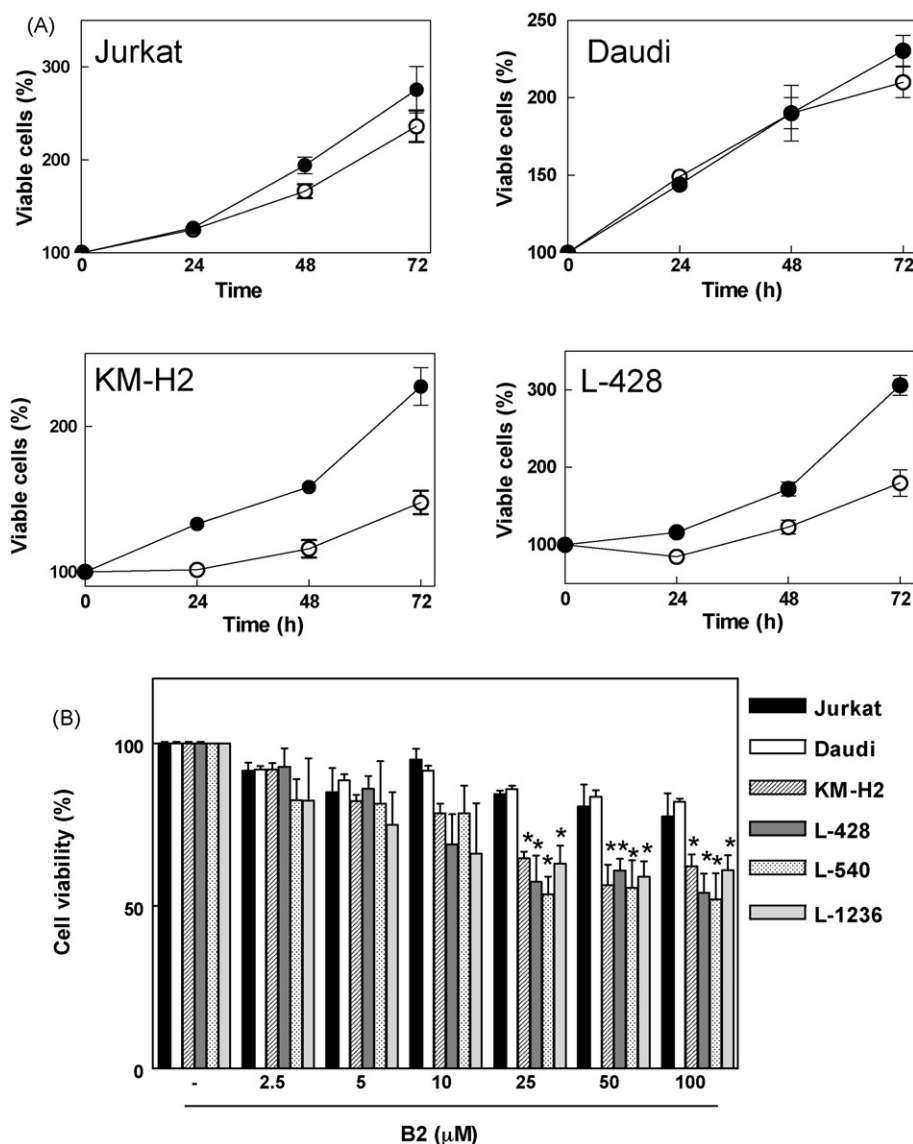
### 3.5. B2 leads to a partial decrease in cell viability only observed in cells with a constitutive activation of NF- $\kappa$ B

The possible effect of B2 on cell viability was next evaluated by measuring the ATP cell content (Fig. 5). We compared the effect of B2 in T and B cells of cancer origin that lack a constitutive activation of NF- $\kappa$ B (Jurkat and Daudi cells) with those on H-RS cells. While B2 did not affect cell viability in Jurkat and Daudi cells, KM-H2 and L-428 cells exhibited a time-dependent decrease in cell viability when cells were incubated with 25  $\mu$ M B2 (Fig. 5A). The effect of different concentrations of B2 (2.5–100  $\mu$ M) on cell viability after 72 h incubation was next investigated in all the cell lines. B2 did not have a significant effect on cell viability in Jurkat and Daudi cells along the range of concentrations tested. On the contrary, B2

**Table 1 – B2 inhibits NF- $\kappa$ B-regulated cytokine production in H-RS cells**

Cell line	B2 (25 $\mu$ M)	IL-6 (pg/ml)	TNF $\alpha$ (pg/ml)	RANTES (pg/ml)
KM-H2	–	249.5 $\pm$ 43.3	98.0 $\pm$ 20.0	3257 $\pm$ 167
KM-H2	+	157.5 $\pm$ 17.8*	38.1 $\pm$ 3.2*	2449 $\pm$ 228*
L-428	–	168.4 $\pm$ 17.0	52.4 $\pm$ 6.7	3113 $\pm$ 438
L-428	+	120.7 $\pm$ 11.0*	24.6 $\pm$ 4.7*	1372 $\pm$ 390*
L-540	–	35.8 $\pm$ 15.2	4.5 $\pm$ 1.1	1372 $\pm$ 99
L-540	+	14.9 $\pm$ 9.9	0.6 $\pm$ 0.4*	864 $\pm$ 177*
L-1236	–	1405.3 $\pm$ 75.2	374.2 $\pm$ 4.8	2120 $\pm$ 97
L-1236	+	1164.6 $\pm$ 30.6*	249.9 $\pm$ 12.6*	1352 $\pm$ 206*
HDLM-2	–	1124.2 $\pm$ 94.3	79.1 $\pm$ 6.2	1553 $\pm$ 80
HDLM-2	+	933.9 $\pm$ 98.6	48.4 $\pm$ 12.7*	956 $\pm$ 55*

KM-H2, L-428, L-540, L-1236 and HDLM-2 cells were incubated for 48 h in the absence or presence of 25  $\mu$ M B2. The concentration of IL-6, TNF $\alpha$  and RANTES released to the media was measured by ELISA. Results are mean  $\pm$  S.E.M. of 3 independent experiments. (\*) Significantly different compared to untreated cells ( $p < 0.05$ , one-way ANOVA test).



**Fig. 5 – Effects of B2 on cells H-RS cell viability. (A) Time-dependent variations in cell viability were measured in Jurkat, Daudi, KM-H2 and L-428 cells after 24–72 h in the absence (full circles) or presence (empty circles) of 25 μM B2. (B) Dose-dependent cell viability was measured in Jurkat, Daudi, KM-H2, L-428, L-540 and L-1236 cells after 72 h in the absence (–) or presence of 2.5–100 μM B2. After quantitation, results are shown as mean ± S.E.M. of 5 independent experiments. (\*) Significantly different compared to untreated cells ( $p < 0.05$ , one-way ANOVA test).**

caused a concentration (2.5–100 μM)-dependent partial decrease in cell viability that reached a maximum effect at 25 μM B2 (35–47% decrease) in all the H-RS cells tested (Fig. 5B).

### 3.6. B2 does not inhibit STAT3 and AP-1 constitutive activations in H-RS cells

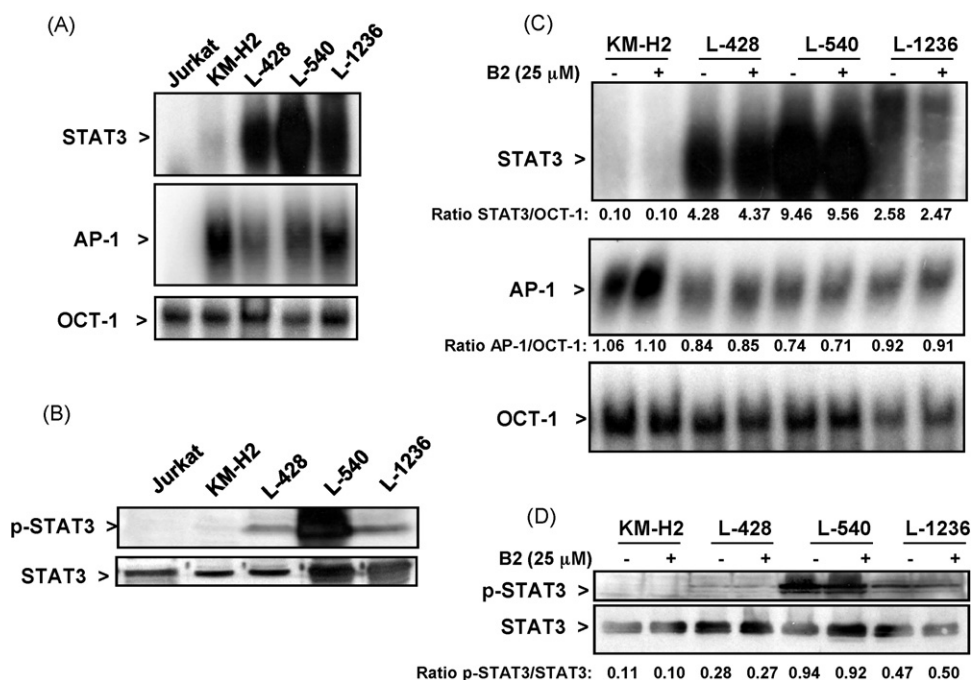
We next evaluated if the partial effect of B2 on cell viability could be due in part to the fact that other signaling pathways overexpressed in H-RS cells may not be affected by B2. Both STAT3 and AP-1 are constitutively active in all the H-RS cells compared to Jurkat T cells, although at a different extent (Fig. 6A). STAT3 phosphorylation was also increased in H-RS cells compared to Jurkat T cells (Fig. 6B). The incubation of KM-

H2, L-428, L-540 and L-1236 cells for 24 h in the presence of B2 did not inhibit either STAT3- or AP-1-DNA binding in nuclear fractions as measured by EMSA (Fig. 6C). Furthermore, STAT3 phosphorylation levels were similar for all H-RS cells when they were incubated in the absence or presence of 25 μM B2 (Fig. 6D).

## 4. Discussion

Constitutive NF-κB activation is a common characteristic of H-RS cells in culture as well as in cells isolated from Hodgkin's patients [5,12]. Therefore, targeting NF-κB pathway emerges as a valuable therapeutic strategy in the treatment of HL. The





**Fig. 6 – B2 does not inhibit STAT3 or AP-1 constitutive activation in H-RS cells. (A)** STAT3-, AP-1- and OCT-1-DNA binding in nuclear fractions isolated from Jurkat, KM-H2, L-428, L-540 and L-1236 cells measured by EMSA. **(B)** Phosphorylated STAT3 (p-STAT3) and STAT3 levels in total fractions isolated from Jurkat, KM-H2, L-428, L-540 and L-1236 cells measured by Western blot. **(C)** STAT3- and AP-1-DNA binding were measured by EMSA in nuclear fractions isolated from L-428, KM-H2, L-540 and L-1236 cells incubated for 24 h in the absence or presence of 25 μM B2. One representative experiment out of 3 independent experiments is shown. **(D)** Phosphorylated STAT3 (p-STAT3) and STAT3 levels were measured by Western blot in nuclear fractions isolated from L-428, KM-H2, L-540 and L-1236 cells incubated for 24 h in the absence or presence of 25 μM B2. One representative experiment is shown.

present results showing that B2 can inhibit constitutive NF-κB activation and NF-κB-regulated anti-apoptotic proteins in H-RS cells suggest that B2 could be of potential use as an inhibitor of NF-κB in HL. On the other hand, given that B2 did not inhibit constitutive AP-1 and STAT3 activation, the moderate effects of B2 on H-RS cell viability may be due to the complex signaling aberrations present in HL. Thus, several signaling pathways should be targeted when designing targeted therapeutics for HL.

The molecular alterations that could lead to NF-κB constitutive activation in H-RS cells are heterogeneous and include: (a) a constitutive high activity of IKK leading to an increased IκBα degradation [5]; (b) mutations in the IκBα [15,16,18] and IκBε genes (10–25%) [17]; (c) amplifications of the c-Rel gene locus (54–80% of patients with classic HL) [21,22]; (d) overexpression of CD30 leading to a CD30-ligand-independent NF-κB activation (100%) [23] and (e) EBV-positive cases (40–50%), where NF-κB activation generally occurs secondary to the expression of the EBV-encoded latent protein 1 (LMP-1) which activates NF-κB [24]. The heterogeneity of the underlying molecular mechanisms leading to NF-κB activation indicates that molecules such as B2, that can inhibit a late event in the NF-κB activation pathway, could be one valuable tool for the targeted treatment of HL.

Reactive oxygen and nitrogen species are recognized triggering signals of NF-κB activation [25,26]. Thus, a con-

stitutive high oxidant production could be a contributing factor to NF-κB activation in H-RS cells. Significantly, all the studied H-RS cells showed high levels of oxidants. Although the underlying mechanisms were not characterized, the binding of several cytokines to their receptors is associated with an increased oxidant production [27] that is attributed to a physiological mechanism of potentiation of cytokine-triggered signaling. No correlations between the levels of cell oxidants and of NF-κB activation were observed among the different H-RS cell lines. This is not unexpected given the differences in the underlying mechanisms of NF-κB activation. B2 acted by partially inhibiting oxidant production in all the H-RS cell lines studied. In L-540 and L-1236 cells that possess the upstream regulatory NF-κB events (NIK-IKK-IκBα), the antioxidant action of B2 could be in part involved in the NF-κB inhibitory action of B2. However, probably due to the high amounts of oxidants produced by L-540 and L-1236 cells or to the involvement of oxidant-unrelated mechanism of NF-κB activation, B2 did not inhibit IκBα phosphorylation and/or prevent IκBα degradation. From the above, the antioxidant capacity of B2 is not a relevant mechanism involved in the inhibition of NF-κB by B2 in H-RS cells.

The finding that PMA or TNFα failed to stimulate NF-κB in H-RS cells indicates that these cells have a maximum level of constitutive NF-κB activation [5]. Nevertheless, B2 was active in inhibiting NF-κB-DNA binding and NF-κB-dependent gene

expression in H-RS cells. B2 inhibited NF- $\kappa$ B-DNA binding in a dose-dependent manner in all the H-RS cells studied and to a similar extent. Interestingly, the inhibition occurred in KM-H2 and L-428 cells, which due to mutations in the inhibitory I $\kappa$ B proteins lack the upstream inhibitory mechanisms. After 24 h incubation with B2, the nuclear content of the NF- $\kappa$ B proteins RelA and p50 was similar both in the absence or presence of B2. Taken together and as previously proposed [14], the above evidence supports a direct inhibition of B2 on the binding of NF- $\kappa$ B to its consensus DNA  $\kappa$ B sites.

H-RS cells express and secrete several cytokines (IL-1 $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, TNF $\alpha$ , TNF $\beta$ , etc.) [28-32]. Some of them are transcriptionally regulated by NF- $\kappa$ B, while others can stimulate NF- $\kappa$ B and contribute to a cycle of NF- $\kappa$ B activation in H-RS cells. Besides the action of cytokines favoring H-RS proliferation and survival, they participate in the activation and chemotaxis of surrounding cells and in the immune deficiency and symptoms that characterize HL patients. In agreement with the observed inhibition of NF- $\kappa$ B-DNA binding activity, B2 also inhibited the release of several NF- $\kappa$ B-regulated cytokines in H-RS cells. Given the aforementioned relevance of cytokines in HL, the inhibition of cytokine production by H-RS cell can be critical in the progression of this disease.

The overexpression of NF- $\kappa$ B-driven genes associated with cell growth and apoptosis have been identified in H-RS cells. They include genes encoding for proteins involved in the regulation of the cell cycle and proteins that prevent apoptosis (Bfl-1/A1, c-IAP2, TRAF1 and Bcl-xL) and cell surface receptors (CD86 and CD40) [30,33]. Treatment with B2 also caused decreased expression of the NF- $\kappa$ B-regulated anti-apoptotic proteins Bcl-xL, Bcl-2, XIAP and cFLIP. Bcl-xL and Bcl-2 are members of the Bcl-2 gene family which protect cells from apoptosis by inhibiting the release of cytochrome c and of Smac/DIABLO from the mitochondria, which inactivates XIAP [34,35]. XIAP and FLIP prevent apoptosis by inhibiting caspases [34,36]. Although B2 partially decreased cell viability, it did not significantly affect the progression of the cell cycle, nor affect parameters of apoptosis within the 72 h of incubation (data not shown). Importantly, B2 did not affect cell viability of Daudi B lymphoblast cells and of Jurkat T leukemia cells, both lacking constitutive NF- $\kappa$ B activation. This indicates the involvement of B2-mediated NF- $\kappa$ B inhibition in the observed decrease in H-RS cell survival and its low toxicity to other cells.

Besides NF- $\kappa$ B, several other signaling pathways are altered and overactivated in HL. For example, STAT3 is constitutively active in HL [37]. In addition, a constitutive and aberrant expression of the AP-1 complex, containing c-Jun and JunB, was identified in patients with classic HL and in H-RS cells [38]. Interestingly, both STAT3 and AP-1 transcription factors have been identified as crucial mediators of both cell cycle promoting and cell-death-inhibiting pathways in H-RS cells [37,38]. We observed that B2 did not inhibit either constitutive STAT3 or AP-1 activation in all the H-RS cells tested. The above could explain the partial effect of B2 on cell viability and suggest that several signaling pathways, especially those involved in cell proliferation and/or resistance to apoptosis, should be targeted when designing therapeutic approaches for HL. However, the capacity of B2 to inhibit NF- $\kappa$ B-regulated

anti-apoptotic proteins could act to decrease the resistance of H-RS cells to undergo apoptosis and therefore make them more susceptible to the action of other chemotherapeutic agents.

It is important to note that the B2 concentrations used in this study represent achievable concentrations for the oral cavity and gut. While the dimer concentrations used are higher than those typically found in blood after the consumption of flavonoid-rich foods, these concentrations can be achieved if the dimer, as a pharmaceutical, is administered intravenously.

In summary, B2 acts by inhibiting constitutive NF- $\kappa$ B activation in H-RS cells including in those bearing I $\kappa$ B mutations. The above evidence indicates that B2 acts preventing the binding of NF- $\kappa$ B to its DNA consensus  $\kappa$ B sequence. This leads to a decreased transactivation of NF- $\kappa$ B-dependent genes involved in the progression of HL. The relevance of other constitutively active signaling cascades (STAT3, AP-1) in H-RS cell proliferation and resistance to apoptosis needs to be established to delineate an efficient targeted treatment of HL.

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