The fully human anti-CD30 antibody 5F11 activates NF-κB and sensitizes lymphoma cells to bortezomib-induced apoptosis

Short title: combined immunotherapy of malignant lymphomas using bortezomib and anti-CD30

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Abstract

5F11, a fully human monoclonal antibody directed against CD30 effectively induces killing of CD30-expressing lymphoma cell lines in vitro and in animal models. A recently conducted phase I/II study shows that 5F11 is well tolerated in heavily pretreated patients with relapsed and refractory CD30-positive lymphoma and has some clinical activity.

In the present study we demonstrate that 5F11 activates NF-kB and the anti-apoptotic protein c-flip in HD (Hodgkin’s lymphoma)-derived cell lines, which might cause apoptosis resistance thus limiting its clinical use. To overcome this resistance we combined 5F11 with the proteasome inhibitor bortezomib which has been shown to suppress NF-kB activity. This combination revealed a synergistic cytotoxic effect in vitro and in a human HD xenograft model provided that 5F11 precedes bortezomib treatment. We conclude that initial 5F11-mediated NF-kB signaling sensitizes the tumor cells to bortezomib-induced cell death. These data suggest a therapeutic value of this combination for HD patients.
Introduction

The CD30 receptor, a member of the tumor necrosis factor superfamily, is selectively overexpressed in the malignant cell population of Hodgkin’s lymphoma (HD) and anaplastic large-cell lymphoma (ALCL) rendering this antigen an excellent target for antibody-based immunotherapy. However CD30 signaling is pleiotropic since antibody binding may induce cell death in ALCL cells, whereas some CD30-positive HD cells respond with growth stimulation.\(^1\)\(^-\)\(^3\). Recently two monoclonal antibodies, the human 5F11 \(^4\) and the humanized SGN-30 \(^5\) exhibited cytotoxicity against HD-derived cell lines as well as against ALCL cells, even though limitations in the sensitivity of HD-target cells were observed. Resistance against apoptosis upon CD30 stimulation might be due to the activation of the survival factor NF-kB, a key anti-apoptosis factor in HD.\(^6\)\(^-\)\(^8\). We found that CD30 stimulation via CD30 activates NF-kB and its target c-flip, which can be inhibited with bortezomib, a proteasome inhibitor known to suppress NF-kB activation. Combination of 5F11 and bortezomib results in cytotoxic synergy both in vitro and in vivo and we conclude that down regulation of NF-kB through bortezomib may be a therapeutic approach to improve CD30 based immunotherapy.
Material and Methods

Cell lines, antibodies, reagents and plasmids
The Hodgkin-derived cell lines (L540 and L428), the ALCL-line Karpas 299 and the CD30-negative acute lymphoblastic leukemia-line REH were described previously ⁹. The anti-CD30 antibody 5F11 was kindly provided by Medarex Inc. (Bloomsbury, USA). Other reagents used were goat anti-human Fc antibody (Dianova/Jackson, USA), the proteasome inhibitor bortezomib (Velcade, Millennium, USA), rabbit-anti p65-IgG and FITC-labeled mouse-anti-rabbit-IgG (Santa Cruz, USA), anti-bcl-2 and anti-bax antibodies (Apoptosis Sampler Kits I and II (BD Bioscience, USA)) and the anti-c-flip antibody (Sigma (F-6550)). NF-kB expression vectors and reporter constructs (NF-kB-luc, IkBαM) were purchased from Clontech, BD Biosciences, USA.

XTT-Assay
The XTT assay was performed as described, ⁴,⁹. Cells were preincubated at 37°C for 30 minutes with 5µg/ml anti-CD30 antibody 5F11 + 25µg/ml goat-anti-human (GaH-IgG) and exposed to increasing concentrations of bortezomib for 48 hours.

 Luciferase reporter gene assay
Transfected L428 cells (electroporation efficacy 5-10 %) were incubated either with 5µg/ml 5F11+ 25µg/ml GaH for 2 hours, or with 50 ng/ml bortezomib or both or plain medium for 24h at 37°C. Luciferase activity was measured as recommended by Promega, USA.

Electrophoretic mobility shift assay
The nuclear protein extracts (20 mM HEPES, pH 7.9, 400 mM NaCl, 1mM EDTA, 1mM EGTA) derived from cells treated with different antibodies were incubated with ³²P-labeled NF-kB binding site oligonucleotides (Santa Cruz) for 30 minutes at room temperature before electrophoresis on 6 % native polyacrylamide gels.

Xenograft model of human HD
The xenograft model of human HD was described previously ⁴. SCID mice with established tumors of about 100mm³ were divided randomly into four groups and received 100 µg 5F11 (in 200 µL PBS intraperitoneally) and 6 hours later 10 ng bortezomib via the tail vein or each agent alone once weekly for a total of 4 injections. Control mice received PBS only. The experiment was stopped and mice were sacrifized when the median tumor diameter in the control group exceeded 2500 mm³ (day 50). Two independent experiments were performed.
Results and Discussion

5F11-dependent CD30 signaling activates NF-kB and c-flip

The comparison of the subcellular distribution of NF-kB in the untreated Hodgkin cell line L540 (fig. 1A) and after incubation with 5F11/GaH (B, C: 6 and 8 hours incubation) shows a nuclear accumulation of NF-kB after treatment, demonstrating its activation in response to CD30 signaling. This activation was inhibited when bortezomib is given 30 minutes after 5F11 stimulation leading to the exclusion of NF-kB from the cell nucleus (shown after 6 and 8 hours, D,E). Similar results were obtained using L428 cells (data not shown). Accordingly, an NF-kB-responsive luciferase reporter gene was activated in L428 cells after stimulation with 5F11 (F). This activation was suppressed in the presence of bortezomib. No luciferase activation was detected upon co-expression of IkBαM, the constitutive active mutant of the NF-kB inhibitor IkB serving as a control. NF-kB DNA binding (G) was detectable in untreated L540 and L428 cells. Exposure to crosslinked 5F11 enhanced DNA binding in both cell lines.

Since a number of factors have been reported to block the apoptotic cascade, we measured the expression level of pro-survival proteins related to NF-kB activation. Most changes were observed for the expression of the caspase inhibitor c-flip which was upregulated by NF-kB. C-flip known to be processed by ubiquitination and proteasomal degradation 10,11 was strongly induced in L540 (H) and L428 cells by 5F11 (data not shown) but was downregulated after co-incubation of the cells with bortezomib. For bcl-2 and bax only minor changes were observed and the expression levels of TRADD and FADD remained unaltered (data not shown). Interestingly c-flip is the key regulator of death receptor resistance in Hodgkin/Reed-Sternberg cells, as down regulation induces autonomous Fas-mediated cell death 12-14.

These data provide evidence that resistance to CD30-mediated cell death in HD cells is due to activation of NF-kB.

Combination of 5F11 and bortezomib increases the cytotoxicity against HD- and ALCL-derived cell lines

We subsequently evaluated the cytotoxic potential of 5F11 to overcome apoptosis resistance when combined with bortezomib in XTT viability assays.

The cell lines tested were sensitive to bortezomib (IC50, 15 ng/ml for L540 cells, 10 ng/ml for Karpas299, 75 ng/ml for L428 cells). Fig. 2A-D presents cell survival upon
exposure to subtoxic concentrations of 5F11 and increasing concentrations of bortezomib. Combining these two reagents resulted in enhanced cell death for L540 and Karpas 299 and interestingly also for L428 cells, which are resistant to 5F11 even at higher antibody concentrations \(^4\). No effect was seen with the lymphoblastic leukemia line REH lacking CD30 expression. The pre-incubation with 5F11 is essential for this synergy since pre-incubation with bortezomib for 30 minutes prior antibody addition had no effect (shown for L428). A similar mechanism was recently reported for leukemic stem cells pretreated with NF-kB-activating anthracyclines before exposure to the proteasomes inhibitor MG132 \(^{15}\).

**In vivo activity of 5F11 and bortezomib in a human Hodgkin model**

L540-derived tumor bearing mice were treated either with 5F11, bortezomib, the combination of 5F11 and bortezomib or PBS. No cross-linking goat-anti-human-antibody was given. Both 5F11 and bortezomib induced significant delayed tumor growth compared to the control (\(P=0.004\) and \(P=0.002\), respectively). Even more promising results were obtained in animals treated with the combination of 5F11 and bortezomib (significant compared to the control (\(P=0.0002\)); 5F11 (\(P=0.0006\)) and bortezomib (\(P=0.0237\)) as the tumor growth was almost completely inhibited (2E, F).

The growth inhibition was maintained for several weeks after treatment, whereas animals receiving either 5F11 or bortezomib alone showed tumor progression.

In conclusion, down regulation of NF-kB through bortezomib after CD30 mediated NF-kB activation may become a therapeutic approach to improve immunotherapy using monoclonal antibodies against the CD30 antigen. These findings are supported by recently published data showing that bortezomib induces cell death irrespective of NF-kB inhibitor mutations frequently observed in HD-derived cells\(^{18}\). Although bortezomib has significant single-agent activity in patients with multiple myeloma and mantle cell lymphoma\(^{19}\) the efficacy in HD patients is less clear. Currently, there are only few patients reported although the responses seem less impressive\(^{21,22}\). The German Hodgkin Study Group is currently evaluating a combination of bortezomib and dexamethasone in relapsed HD patients. Depending on the outcome of this phase-II-trial the preclinical data presented provide a rationale for a clinical study combining bortezomib/dexamethasone with an anti-CD30 monoclonal antibody.
References


Legends

Figure 1
Regulation of NF-κB distribution and activity by 5F11 and bortezomib
Detection of the NF-κB subunit p65 with anti-p65 and a secondary FITC-labeled antibody in untreated L540 cells (A), after stimulation with crosslinked 5F11 for 6 and 8 hours (B,C) and bortezomib (D,E). The cell nuclei are stained with DAPI. Pictures were made using the digital Nikon Eclipse E800 microscope with the LuciaG/F program. A: 10xocular, B-E: 40xocular.

(F) L428 cells were transfected with either reporter construct pCMV-luc (transfection control) or pNF-κB-luc (bar 2-4) or pNF-κB-luc together with the expression vector IκBαM (bar 5-7) and exposed to PBS (basal), 5F11 or bortezomib for 24 hours. The luciferase activity (RLU) of the cell lysates is indicated.

(G) EMSA to detect DNA binding of NF-κB in nuclear extracts of L540 and L428 cells after 6 hours incubation with no antibody, cross-linked 5F11 and cross-linked Ber-H2 (BH2) antibody. Ber-H2 as a control anti-CD30 antibody is unable to mediate CD30 signaling and fails to activate NF-κB.

(H) Western blotting of whole L540 cell extracts to detect c-flip, bcl-2 and bax in untreated cells after 16 hours incubation (n.t for no treatment) and following treatment with either 5F11 or 5F11 + bortezomib after 6, 8 and 16 hours incubation.

Figure 2
Cytotoxic synergy of 5F11 in combination with bortezomib
(A-D) XTT viability assays of various cell lines after 48-hours exposure to subtoxic concentrations of cross-linked 5F11 (5 µg/ml 5F11; 25 µg/ml GaH-IgG), increasing concentrations of bortezomib (○) and the combination of both (●). The cell viability after exposure to 5 µg/ml 5F11 (△), 25 µg GaH (▽) and same amounts of 5F11+GaH (◆) is indicated. The mAb 5F11 and GAH-IgG (goat anti-human immunoglobulin G) were distributed in 100-µl aliquots in 96-well plates with target cells (2 x 10⁴; L540, Karpas299, L428, REH) and the plates were incubated for 30 minutes before addition of bortezomib. A synergistic increase of cytotoxicity is observed for the CD30 expressing cell lines (A-C). The synergy is not seen upon pre-incubation with bortezomib before 5F11/GaH addition (C, ◇) and for the CD30 negative cell line REH (D). Means and standard deviation of three independent experiments are given.
E) Depiction of the XTT-data for subtoxic concentrations of cross-linked 5F11 (5F11/GaH: 5/25 µg/ml for each cell line), subtoxic concentrations of bortezomib (L540: 7.5 µg/ml; Karpas299: 2.5 µg/ml; L428: 50 µg/ml; REH: 5 µg/ml) and the combination of both. (F) Effect of 5F11 and bortezomib on the tumor growth of subcutaneous L540Cy Hodgkin tumors in SCID mice. One of two independent experiments is shown and the standard error mean is given. (G) Statistical analysis of the tumor volumes measured on day 47 (see F, and an independent serie, each group with n=4). The differences between 5F11, bortezomib and the combination versus the control are significant (the $P$ values are estimated with the paired t-test using GraphPadPrism software).
Figure 1, Böll et al.
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