Combining Milatuzumab with Bortezomib, Doxorubicin, or Dexamethasone Improves Responses in Multiple Myeloma Cell Lines

Rhona Stein,1 Mitchell R. Smith,2 Susan Chen,1 Maria Zalath,1 and David M. Goldenberg1

Abstract

Purpose: The humanized anti-CD74 monoclonal antibody, milatuzumab, is in clinical evaluation for the therapy of multiple myeloma (MM). The ability of milatuzumab to increase the efficacy of bortezomib, doxorubicin, and dexamethasone was examined in three human CD74+ MM cell lines, CAG, KMS11, KMS12-PE, and one CD74-MM cell line, OPM-2.

Experimental Design: Activity of milatuzumab as a monotherapy and combined with the drugs was evaluated by studying in vitro cytotoxicity, signaling and apoptotic pathways, and in vivo therapeutic activity in severe combined immunodeficient (SCID) mouse models of MM.

Results: Given as a monotherapy, cross-linked milatuzumab, but not milatuzumab alone, yielded significant antiproliferative effects in CD74+ cells. The combination of cross-linked milatuzumab with bortezomib, doxorubicin, or dexamethasone caused more growth inhibition than either cross-linked milatuzumab or drug alone, producing significant reductions in the IC50 of the drugs when combined. Efficacy of combined treatments was accompanied by increased levels of apoptosis measured by increases of activated caspase-3 and hypodiploid DNA. Both milatuzumab and bortezomib affect the nuclear factor-κB pathway in CAG MM cells. In CAG- or KMS11-SCID xenograft models of disseminated MM, milatuzumab more than doubled median survival time, compared with up to a 33% increase in median survival with bortezomib but no significant benefit with doxorubicin. Moreover, combining milatuzumab and bortezomib increased survival significantly compared with either treatment alone.

Conclusions: The therapeutic efficacies of bortezomib, doxorubicin, and dexamethasone are enhanced in MM cell lines when given in combination with milatuzumab, suggesting testing these combinations clinically.

Multiple myeloma (MM) is the second most prevalent blood cancer after non–Hodgkin lymphoma (1, 2). It represents ~1% of all cancers and 2% of all cancer deaths. Approximately 50,000 Americans currently have myeloma, and the American Cancer Society estimates 19,920 new cases of myeloma and 10,690 deaths in 2008. Currently available therapies for myeloma include chemotherapy and stem cell transplantation, as well as new emerging therapies and combination therapy regimens that are being tested in clinical trials (2, 3). High-dose melphalan followed by autologous stem cell transplantation is currently the standard of care because of its high response rate and relatively low morbidity and mortality. Because stem cell transplantation is neither curative nor applicable to all patients and improved complete response rates have been seen in recent clinical trials, the National Comprehensive Cancer Network issued treatment guidelines recently that include recommendations for the use of newer treatment options for myeloma patients. Lenalidomide and bortezomib regimens involving combination therapy with doxorubicin and/or dexamethasone are now recommended in initial therapy protocols for transplant candidates and for treatment of relapsed/refractory disease. Combination of melphalan and prednisone with thalidomide or bortezomib are recommended as initial therapy for nontransplant candidates. Thus, there has been considerable progress in the field of myeloma therapy. Although cures have not been documented, molecular complete responses have been achieved with some of the new therapies. However, relapses still occur after molecular complete response, usually after a longer period of event-free survival.

Despite the substantial progress, the unmet need for improved therapy of MM has led to the evaluation of cell surface targets expressed by the myeloma cells. Although antigens such as CD19 and CD20 are important targets for non–Hodgkin lymphoma, they are expressed in only a minority of myeloma cases. In contrast, the cell surface protein CD74 is expressed at fairly high levels in nearly 90% of MM clinical specimens but not on most normal human cells (4). In addition, the humanized anti-CD74 monoclonal antibody, milatuzumab (hLL1), has in vitro growth inhibitory effects on
MM cell lines and therapeutic effects in MM models (5, 6). Based on these preclinical studies, milatuzumab is currently in clinical evaluation for therapy of MM. Here, we provide in vitro and in vivo data corroborating the therapeutic efficacy of milatuzumab in human MM model systems and expand our observations to show that this monoclonal antibody is more effective than key therapeutic agents for MM treatment, including bortezomib, doxorubicin, and dexamethasone when added (100 μL) per well (100 μL) in 96-well plates, to which antibodies and/or drugs were added (100 μL). After incubation for 4 d at 37°C in a humidified CO₂ (5%) incubator, 25 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added, and the cells were incubated for an additional 4 h at 37°C. Plates were then centrifuged and supernatants were removed. Pellets were dissolved using 100 μL DMSO per well and absorbance was measured at 570 nm on a microplate reader ( Molecular Devices). Because unlabeled milatuzumab was reported previously to require cross-linking for cytotoxic activity (6), goat anti-human IgG (GAH) was added to some of the wells. Milatuzumab was used at a final concentration of 5 μg/mL and GAH was used at 20 μg/mL. Percent growth inhibition and IC₅₀ values were determined using 4 replicates.

DNA fragmentation. Flow cytometric analysis of cellular DNA was done after propidium iodide staining (11, 12). Cells were placed in 24-well plates (1.5 to 3 x 10⁵ cells per well) and treated with drugs (at concentrations indicated for each experiment) and/or mAbs (5 μg/mL) in the presence or absence of a second antibody (20 μg/mL). Percent apoptotic cells (hypodiploid cells) was determined after a 48-h incubation.

Cleaved caspase-3. Cells were incubated in the presence or absence of the drugs (at concentrations indicated for each experiment) and/or mAbs for 48 h. Changes in the intracellular levels of cleaved caspase-3 were measured using FITC-conjugated rabbit anti-activated caspase-3 (BD Bioscience) as per the manufacturer’s directions. Analyses were done on the FACSCalibur.

Western blots. Cells were cultured in the presence or absence of the mAbs and/or drugs for the indicated times, pelleted, washed thrice in PBS, then lysed in ice cold radioimmunoprecipitation assay buffer containing 100 μg/mL phenylmethylsulfonyl fluoride and 1 μg/mL aprothin on ice for 30 min. Bortezomib was used at 1 ng/mL. Milatuzumab was used at a final concentration of 5 μg/mL and GAH was used at 20 μg/mL. The lysates were centrifuged at 1,200 x g for 20 min. The samples were then electrophoresed on a 4 to 12% Bis-Tris gel (Invitrogen) and transferred to PVDF membranes. The membranes were blocked in 5% BSA in PBS-T (20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.05% Tween 20) for 1 h at RT, washed 3x with PBS-T, and incubated with the respective antibodies at 4°C overnight. The next day, the membranes were washed 3x with PBS-T and incubated with appropriate HRP conjugated secondary antibodies for 1 h at RT. Membranes were washed and developed using Amersham ECL reagent and imaged on a Fujifilm LAS-3000 reader.

**Table 1. Antigen expression on MM cell lines**

<table>
<thead>
<tr>
<th>Control-Ag8 % Positive (geometric mean FL)</th>
<th>CD20-2B8</th>
<th>CD138-B-B4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG</td>
<td>54 (6.7)</td>
<td>4.4 (6.0)</td>
</tr>
<tr>
<td>KMS11</td>
<td>2.2 (4.9)</td>
<td>2.1 (4.9)</td>
</tr>
<tr>
<td>KMS11-PE</td>
<td>1.8 (2.4)</td>
<td>2.7 (2.3)</td>
</tr>
<tr>
<td>OPM-2</td>
<td>1.4 (5.4)</td>
<td>1.2 (5.3)</td>
</tr>
</tbody>
</table>

**NOTE:** Antigen expression was determined by flow cytometric analyses.

**Fig. 1.** Effects of milatuzumab and GAH second antibody on proliferation of cell lines. Anti-proliferative effects of milatuzumab were determined by MTT assays. Cells were cultured with 5 μg/mL milatuzumab with or without 20 μg/mL second antibody for cross-linking to mimic the role of effector cells or cross-linking molecules present in vivo. Percent inhibition is calculated relative to untreated control cells. Columns, mean of four replicates; bars, SD. *p not determined.
15 min at 4°C. Supernatants were removed and separated on 10% to 20% SDS-PAGE (Tris-HCl, ReadyGel; Bio-Rad) followed by transfer to Immobilon-P membrane (Millipore). Membranes were blocked with 5% nonfat dry milk-0.05% Tween20 (Sigma-Aldrich Corp.) in PBS for 1 h and then incubated overnight in the presence of antibody at 4°C. Primary antibodies were from Cell Signaling Technology and were used at 1:1,000 dilution, except Bid from Trevigen used at 1:1,000, and Mcl-1 from BD Pharmingen and Bcl-2 from Santa Cruz Biotechnology used at 1:500. The blots were incubated in secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) for 1 h, followed by chemiluminescent detection (Amersham).

**In vivo studies in severe combined immunodeficient mice bearing disseminated MM tumors.** For studies on the therapeutic effect of the mAbs and drugs in CAG-bearing severe combined immunodeficient (SCID) mice (female, 6- to 8-wk-old; Charles River Laboratories), the mice were immunosuppressed by pretreatment with fludarabine and cyclophosphamide 3 days before i.v. injection of 5 to 10 \( \times 10^6 \) tumor cells, as previously described (6). Pretreatment with fludarabine and cyclophosphamide was not used for implantation of KMS11 or KMS12-PE. Dosing is described in figure legends. Mice were examined daily for signs of distress or hind leg paralysis, and weighed weekly. Paralysis of the hind legs or a weight loss of >20% was used as the survival end point. Animals were euthanized at these end points. Experiments used groups of 8 to 10 mice. Animal studies were done under protocols approved by the Institutional Animal Care and Use Committee.

**Statistical considerations.** For *in vitro* studies, means and SDs were calculated. Differences between treatment groups were compared using the Student's *t* test. In *in vivo* studies, overall survival was evaluated graphically by the methods of Kaplan and Meier. Pair-wise comparisons between specific groups were made using log-rank statistics. Significance for all tests was declared at \( P \) values of <0.05.

### Results

**In vitro cytotoxicity of milatuzumab on MM cell lines.** The cytotoxicity of milatuzumab was investigated on cell lines derived from MM patients, alone or cross-linked with GAH. CAG, KMS11, and KMS12-PE are positive for CD138 (syndecan-1) and CD74, and CD20 negative. OPM-2 is CD138 positive but does not express cell surface CD74 or CD20. Antigen expression of these cell lines is tabulated in Table 1. As shown in Fig. 1, the CD74+ cell lines are sensitive to killing by cross-linked milatuzumab but not milatuzumab in the absence of cross-linking, or the cross-linking antibody (GAH) alone. For example, in the representative data shown, milatuzumab+GAH yielded 38.0% ± 8.9% inhibition (\( P = 0.0025 \)) in CAG cells compared with 5.5% ± 12.8% inhibition in OPM-2 cells (\( P = 0.54 \)) relative to untreated cells. The absence of cytotoxicity of cross-linked milatuzumab in OPM-2, which is CD74-, shows

**Table 2. Combining milatuzumab with drugs in MM cell lines**

<table>
<thead>
<tr>
<th>Drug</th>
<th>CAG IC50 (ng/mL)</th>
<th>KMS11 IC50 (ng/mL)</th>
<th>KMS12-PE IC50 (ng/mL)</th>
<th>OPM-2 IC50 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>7.4 ± 2.1 &amp; 0.0063</td>
<td>1.1 ± 0.7</td>
<td>16.7 ± 3.6 &amp; 0.0027</td>
<td>62.1 ± 2.6 &amp; 0.0205</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>0.40 ± 0.08 &amp; 0.0015</td>
<td>0.12 ± 0.06</td>
<td>0.45 ± 0.04 &amp; 0.0002</td>
<td>1.36 ± 0.04 &amp; 0.0006</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>20.038 ± 550 &amp; 0.0059</td>
<td>145 ± 86</td>
<td>&gt;40,000 *</td>
<td>&gt;40,000 *</td>
</tr>
</tbody>
</table>

NOTE: IC50 values were determined using MTT cytotoxicity assays. Cells were incubated with the indicated drugs in the absence (-) or presence (+) of milatuzumab and GAH for 4 d.
Abbreviation: ND, not determined.

*IC50 value were not calculated because the combination of milatuzumab plus GAH yielded ≥50% inhibition of proliferation relative to untreated cells in the absence of drug.
specificity. Thus, cross-linked milatuzumab specifically and effectively kills CD74+ MM cells in vitro.

In vitro cytotoxicity of bortezomib, dexamethasone, and doxorubicin with and without milatuzumab. MTT assays were done to examine the effects of the drugs, alone and combined with milatuzumab or milatuzumab+2nd antibody (GAH). Representative titrations are shown in Fig. 2 for CAG (data not shown for KMS11, KMS12-PE, and OPM-2). Results

Fig. 3. Apoptotic effect of mAbs on MM cell lines. Induction of apoptosis was evaluated by flow cytometry determination of (A) hypodiploid DNA and (B) activated caspase-3. Cells were incubated with drugs alone, milatuzumab alone, or combinations of drug+milatuzumab with and without a second antibody for cross-linking for 48 h. For determination of hypodiploid DNA, staining was done with propidium iodide. Changes in the intracellular levels of cleaved caspase-3 were measured using FITC-conjugated rabbit anti-activated caspase-3. Analyses were done on the FACSCalibur. ○, solid line, drug only; Δ, dashed line, drug+milatuzumab; □, dotted line, drug+GAH; ●, solid line, drug+milatuzumab+GAH. Columns, mean of three replicates; bars, SD (some points are hidden by the symbol).
DNA by these drugs, with increases by the drugs alone remaining within 12% of baseline. In all cell lines tested, milatuzumab that was not cross-linked with second antibody did not affect the percent of hypodiploid DNA induced by the drug alone, percentages were generally within 1% to 5% of the drug only values. Similar results were obtained in the controls when second antibody was given in the absence of milatuzumab. However, milatuzumab + GAH led to statistically significant increased levels of hypodiploid DNA (P < 0.05). For example in CAG, levels of sub-G0 DNA were 8.4% ± 0.3%, 8.0% ± 0.3%, and 9.0% ± 0.5% after treatment with 0.1 ng/ml bortezomib alone, 0.1 ng/ml bortezomib + milatuzumab, and 0.1 ng/ml bortezomib + GAH, respectively (no significant difference; P > 0.05). However, 0.1 ng/ml of bortezomib + milatuzumab + GAH increased sub-G0 DNA to 34.5% ± 0.3% (P < 0.00001, versus drug alone).

Induction of activated caspase-3 was similarly analyzed by flow cytometry after incubation with increasing doses of bortezomib and doxorubicin. Induction of activated caspase-3 was observed (Fig. 3B). KMS12-PE was the least sensitive cell line in this test. Cross-linked milatuzumab yielded statistically significant increases in the induction of activated caspase-3 in CAG and KMS11. For example, in KMS11 levels of activated caspase-3 were 11.4% ± 0.6%, 8.0% ± 0.7%, and 13.1% ± 0.3% after treatment with 0.1 ng/ml bortezomib alone, 0.1 ng/ml bortezomib + milatuzumab, and 0.1 ng/ml bortezomib + GAH, respectively. However, 0.1 ng/ml of bortezomib + milatuzumab + GAH yielded a value of 41.6 ± 2.1% (P = 0.0009, versus drug alone).

Increases were not seen in OPM-2 and KMS12-PE, presumably due to the lack of CD74 in OPM-2 and the low caspase-3 induction by the drugs in KMS12-PE. These data show that doxorubicin and bortezomib induce apoptosis, as measured here by induction of hypodiploid DNA and activated caspase-3. Moreover, cross-linked milatuzumab amplifies this effect, corroborating the benefit of using these agents in combination.

We have begun to examine the intracellular interactions of antibody and drug by investigating signaling and apoptotic pathways in CAG cells using Western blot analyses. No alterations in Bid, Bax, Bcl-2, nor Mcl-1 were observed after exposure of CAG cells to cross-linked milatuzumab (data not shown). Thus, signaling through the extrinsic, but not the mitochondrial, apoptotic pathways is suggested.

CD74 activation by its ligand macrophage migration-inhibitory factor signals through nuclear factor-κB (NF-κB) in chronic lymphocytic leukemia cells (13). Moreover, stimulation of chronic lymphocytic leukemia cells with C-16, an agonistic anti-CD74 mAb, leads to NF-κB activation, and increased survival in chronic lymphocytic leukemia cells. Here, we find decreases in phosphorylated IκBα and phosphorylated NF-κB 30 minutes after incubation of CAG cells with cross-linked milatuzumab, with recovery of phosphorylated NF-κB to control levels at 3 hours (Fig. 4). In contrast, bortezomib increased phosphorylated IκBα and phosphorylated NF-κB at 3 hours, a result in contrast to the expected decreases in these values. The combination of cross-linked milatuzumab plus bortezomib yielded an intermediate level of phosphorylated NF-κB at 30 min. These data show that both milatuzumab binding to CD74 and bortezomib treatment induce intracellular alterations of the NF-κB signal pathway in CAG.
myeloma cells, although in opposite directions. Future studies examining total IKKα levels (e.g., by electrophoretic mobility shift assays) are necessary to clarify the observed effects on the NF-κB pathway under the various treatment conditions.

In vivo therapeutic activity of milatuzumab as a monotherapy and combined with drugs. A dose-response therapy study in mice bearing the CAG cell line is shown in Fig. 5A and Table 3. Milatuzumab was given at 30, 100, and 300 μg injection, twice weekly for 4 weeks, starting 1 day after injection of CAG cells. Animals were monitored for survival and hind leg paralysis, and body weights were measured weekly. Results of this study showed that treatment with milatuzumab yielded a significant survival benefit in this model. Median survival times (MST) in both untreated CAG-bearing mice and mice given an unreactive isotype control mAb were 42 days. The median survival in the milatuzumab-treated groups was at least doubled, ranging between 84 and 103 days (P < 0.0001 versus untreated). A dose-response was not observed in this dose range.

The effects of combining doxorubicin or bortezomib with milatuzumab were evaluated in SCID mice bearing CAG, KMS11, and KMS12-PE cells. Bortezomib dosing was selected from reports of maximum tolerated dose values in SCID mice (14). Treatments were given as 2 i.p. doses/week for 3 weeks, initiated on day 5 after injection of tumor cells. Figure 5B and Table 4 show the tumor growth curves for CAG-bearing SCID mice treated with bortezomib and milatuzumab. Given as a single agent, 1.0 mg/kg bortezomib caused an apparent treatment-related death of 1 of 10 mice 12 days after administration of the drug, and given in combination with 100 μg milatuzumab, 2 of 10 mice died of treatment-related death (days 8 and 13). The 0.5 mg/kg dose was well-tolerated with no loss of body weight. Median survival in untreated control mice in this study was 33 days. Bortezomib alone increased MST to 40 and 44 days, representing MST increases of 21.2% (P < 0.0001) and 33.3% (P = 0.0021) at 0.5 and 1.0 mg/kg, respectively. Treatment with milatuzumab (100 μg per mouse) increased the MST to 73 days. When bortezomib and milatuzumab treatments were combined, MSTs were increased further to 79 and 93 days for 0.5 and 1.0 mg/kg bortezomib, respectively (P = 0.0441 and P = 0.0065 for the 100 μg milatuzumab + 1.0 mg/kg bortezomib combination versus milatuzumab alone and 1.0 mg/kg bortezomib alone, respectively).

The tumor growth curves for KMS11-bearing SCID mice treated with bortezomib and milatuzumab are shown in Fig. 5C and Table 5. Based on the results of the CAG study, bortezomib was given at 0.8 mg/kg/dose. Milatuzumab was administered at 2 dose levels, 35 and 100 μg/dose. The 0.8 mg/kg dose was well-tolerated with no loss of body weight. Median survival in untreated KM11 control mice in this study was 54 days. Bortezomib alone increased MST to 61 days (P = 0.0667 versus untreated), representing a MST increase of 13.0%. Treatment with 35 μg milatuzumab alone also increased MST to 61 days (P = 0.1518 versus untreated). MST was increased further to 86 days for the combination of
35 μg milatuzumab and 0.8 mg/kg bortezomib, representing a survival increase of 59.3% relative to untreated mice (P < 0.0001). The MST increase due to combination of 35 μg milatuzumab and 0.8 mg/kg bortezomib was statistically significant compared with untreated and both single agent treatment groups (P = 0.0040 and P = 0.0128 for the 35 μg milatuzumab+0.8 mg/kg bortezomib combination versus milatuzumab alone and bortezomib alone, respectively). There were 5 long-term survivors (>4 months) in the 35 μg milatuzumab+0.8 mg/kg bortezomib treatment group, compared with 1 or none in the control and single agent groups at these doses. Increasing the milatuzumab dose to 100 μg/dose increased its effect. Median survival was not reached at 4 months; there were 7 long-term survivors each with 100 μg milatuzumab, and 100 μg milatuzumab+0.8 mg/kg bortezomib.

KMS12-PE is a slower growing tumor than CAG or KMS11. Median survival in untreated KMS12-PE–bearing SCID mice is 110 days (survival curve not shown). At 6 months posttumor injection, 2 of 9 mice were still alive in the untreated group, compared with 3 of 9 given 0.8 mg/kg bortezomib (124 days median survival), 3 of 9 given 100 μg milatuzumab (110 days median survival), and 6 of 9 given the combined treatment (MST not reached at 180 days). Thus, the combined treatment yielded a minimum increase in median survival of 64%. Statistical significance was not reached in this study, presumably due to the slow progression of tumor in this model. P values were 0.0962, 0.2126, and 0.1603 for milatuzumab+bortezomib versus untreated, bortezomib alone, and milatuzumab alone, respectively.

Thus, when given as a monotherapy, milatuzumab is more effective than bortezomib in the CAG and KMS11 MM models. Moreover, combining milatuzumab and bortezomib increases survival compared with either treatment alone in the CAG, KMS11, and KMS12-PE models. Importantly, these studies confirm the therapeutic efficacy of milatuzumab previously reported in MC/CAR-bearing SCID mice (6) in additional MM cell lines.

The efficacy of milatuzumab also was compared with that of doxorubicin and a mixture of doxorubicin and milatuzumab in CAG-bearing SCID mice. Doxorubicin was administered at its maximum tolerated dose in this model, 35 μg, and at one-fourth the maximum tolerated dose, 8.65 μg. Treatments were given as a single i.p. dose 5 days after injection of tumor cells. Treatment with a single dose of milatuzumab (341 μg per mouse) increased the MST from 33 to 69 days, with no significant difference from the milatuzumab plus doxorubicin group, or the 100 μg milatuzumab/mouse twice weekly for

### Table 3. Therapeutic efficacy of bortezomib plus milatuzumab in MM-bearing SCID mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Median survival (d)</th>
<th>P vs untreated</th>
<th>% MST increase*</th>
<th>P vs 100 μg milatuzumab</th>
<th>% MST increase †</th>
<th>P vs corresponding bortezomib concentration</th>
<th>% MST increase ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 μg milatuzumab</td>
<td>91</td>
<td>&lt;0.0001</td>
<td>116.7%</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>100 μg milatuzumab</td>
<td>103</td>
<td>&lt;0.0001</td>
<td>145.2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 μg milatuzumab</td>
<td>84</td>
<td>&lt;0.0001</td>
<td>100.0%</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>300 μg hMN-14</td>
<td>42</td>
<td>0.6820</td>
<td>0.0%</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

NOTE: Statistical summaries for data shown in Fig. 5. CAG-bearing SCID mice were given milatuzumab at 30, 100, and 300 μg/injection, twice weekly for 4 wk, starting 1 d after injection of the CAG cells. Control animals were either left untreated or given hMN-14 (isotype control mAb at 300 μg/injection by the same schedule). Survival data are plotted in Fig. 5A. *Relative to untreated control group.

### Table 4. CAG-bearing SCID mice were treated with bortezomib, milatuzumab, and mixtures of milatuzumab + bortezomib

<table>
<thead>
<tr>
<th>Group</th>
<th>Median survival (d)</th>
<th>P vs untreated</th>
<th>% MST increase*</th>
<th>P vs 100 μg milatuzumab</th>
<th>% MST increase †</th>
<th>P vs corresponding bortezomib concentration</th>
<th>% MST increase ‡</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>33</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/kg bortezomib</td>
<td>40</td>
<td>&lt;0.0001</td>
<td>21.2%</td>
<td></td>
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<td></td>
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<tr>
<td>1.0 mg/kg bortezomib</td>
<td>44</td>
<td>&lt;0.0021</td>
<td>33.3%</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>100 μg milatuzumab</td>
<td>73</td>
<td>&lt;0.0001</td>
<td>121.2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg milatuzumab + 0.5 mg/kg bortezomib</td>
<td>79</td>
<td>&lt;0.0001</td>
<td>139.4%</td>
<td>0.3110</td>
<td>8.2%</td>
<td>&lt;0.0001</td>
<td>97.5%</td>
</tr>
<tr>
<td>100 μg milatuzumab + 1.0 mg/kg bortezomib</td>
<td>93</td>
<td>&lt;0.0238</td>
<td>181.8%</td>
<td>0.0441</td>
<td>27.4%</td>
<td>&lt;0.0065</td>
<td>132.5%</td>
</tr>
</tbody>
</table>

NOTE: Treatments were given as 2 i.p doses/wk for 3 wk, initiated on day 5 after injection of tumor cells. Survival data are plotted in Fig. 5B. *Relative to untreated control group. † Relative to 100 μg milatuzumab group. ‡ Relative to corresponding bortezomib concentration group.
3 weeks dose schedule, shown in Fig. 5B. Given alone, doxorubicin yielded little or no effect on survival compared with untreated animals (data not shown).

**Discussion**

CD74 (invariant chain, Ii) is a type-II transmembrane glycoprotein that associates with the major histocompatibility class II α and β chains and directs the transport of the αβIi complexes to endosomes and lysosomes (15–17). Because the chaperone function of CD74 was thought to be its primary function, it was not anticipated that an anti-CD74 mAb would possess growth-inhibitory activity. However, it is now understood that CD74 is the cellular receptor for the proinflammatory cytokine, macrophage migration-inhibitory factor (18), and that the binding of macrophage migration-inhibitory factor to cell surface CD74 initiates a signaling cascade resulting in proliferation and survival (19). Shachar and coworkers (20) have shown that after migration-inhibitory factor binding to the CD74 extracellular domain, the cytosolic region of CD74 is cleaved by a two-step process. The cytosolic fragment then translocates to the cell nucleus, resulting in activation of NF-κB (21), and induction of a survival cascade in which interleukin-8 (IL-8) levels are elevated, as well as levels of the antiapoptotic protein, Bcl-2 (13). In addition, Helicobacter pylori was shown to bind CD74 on gastric epithelial cells and stimulate NF-κB and IL-8 production, thus corroborating the involvement of these factors in the signal cascade initiated by CD74 activation (22, 23). Milatuzumab apparently acts as an antagonistic antibody, blocking this signal cascade by binding to cell-surface CD74. Using B-CLL patient specimens, Binsky et al. (13) showed that milatuzumab specifically blocked macrophage migration-inhibitory factor–induced up-regulation of Bcl-2 mRNA levels, inhibited IL-8 transcription, and increased the percentage of apoptotic cells.

The humanized anti-CD74 monoclonal antibody, milatuzumab, causes specific growth inhibition and induction of apoptosis in B-cell lines in the presence of a second cross-linking antibody. In addition, significant survival extensions were observed in non–Hodgkin lymphoma– and MM-bearing SCID mice treated with naked milatuzumab without the need for an exogenous cross-linking agent (6). Thus, cross-linking agents are necessary for milatuzumab activity in *in vitro* studies but are not required for milatuzumab activity *in vivo*. Cross-linking with secondary antibodies is believed to function by generating stronger and/or more sustained signals than simple ligation of surface antigens with primary mAbs to the antigens alone (12). A requirement for extensive cross-linking of surface molecules has been noted by several groups studying lymphoma cell growth inhibition, cell cycle arrest, and/or apoptosis *in vitro*. Specifically, hypercross-linking with secondary antibodies has been used for *in vitro* evaluations of anti-CD20, anti-CD19, anti-CD22, anti–major histocompatibility class II, anti-sIgM, and anti–APO-1 mAbs (12, 24–27). However, we and others have observed that cross-linking by GAH is not needed for *in vivo* activity. Presumably the function of the secondary antibodies in *in vitro* is fulfilled *in vivo* by other molecules present in the cellular environment. These may include cross-linking with Fc-receptor–expressing cells (e.g., macrophages, monocytes, or dendritic cells), or other molecules involved in the signaling pathway of the target antigen that cause further aggregation after preliminary stimulation by the primary mAb.

Based on the preclinical evidence of activity, milatuzumab is currently in clinical evaluation for therapy of MM, non–Hodgkin lymphoma, and chronic lymphocytic leukemia. In this article, we showed the ability of milatuzumab to increase the efficacy of bortezomib, doxorubicin, and dexamethasone in MM cell lines. In *vitro*, cross-linked milatuzumab yielded significant cytotoxicity on CD74-positive MM cell lines when given as a single agent and caused significant reductions in the IC50 values of the anti-MM drugs. In *vivo*, milatuzumab given as a monotherapy was more effective than bortezomib or doxorubicin, whereas the combination of milatuzumab and bortezomib was more effective than either agent alone.

Bortezomib is a proteasome-inhibitor, whose mechanism of action is partly mediated through NF-κB (reviewed in refs. 28, 29). NF-κB is constitutively active in MM as well as other tumors and, by influencing transcription of multiple gene targets, supports proliferation and suppresses apoptosis. However, differential responses of NF-κB to bortezomib have been noted in MM as well as other hematologic and nonhematologic cancers and may contribute to heterogeneous responses to bortezomib therapy. Failure of bortezomib to decrease NF-κB

**Table 5.** KMS-11-bearing SCID mice were treated with bortezomib, milatuzumab, and mixtures of milatuzumab + bortezomib

<table>
<thead>
<tr>
<th>Group</th>
<th>Median survival (d)</th>
<th>P vs untreated</th>
<th>% MST increase</th>
<th>P vs corresponding milatuzumab concentration</th>
<th>P vs bortezomib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 mg/kg bortezomib</td>
<td>61</td>
<td>0.0667</td>
<td>13.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg milatuzumab</td>
<td>&gt;125</td>
<td>&lt;0.0001</td>
<td>131.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg milatuzumab + 0.8 mg/kg bortezomib</td>
<td>&gt;125</td>
<td>&lt;0.0001</td>
<td>131.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 μg milatuzumab</td>
<td>61</td>
<td>0.1518</td>
<td>13.0%</td>
<td>0.9542</td>
<td>8.2%</td>
</tr>
<tr>
<td>35 μg milatuzumab + 0.8 mg/kg bortezomib</td>
<td>86</td>
<td>&lt;0.0001</td>
<td>59.3%</td>
<td>0.0040</td>
<td>27.4%</td>
</tr>
</tbody>
</table>

Note: Treatments were given as 2 i.p. doses/wk for 3 wk, initiated on day 5 after injection of tumor cells. Survival data are plotted in Fig. 5C.

a Relative to untreated control group.
b Relative to 0.8 mg/kg bortezomib group.
c Relative to 35 μg milatuzumab group.

1 Relative to 0.8 mg/kg bortezomib group.
activation or to actually cause increased NF-κB activation have been recently reported in MM (30), mantle cell leukemia (31), and endometrial (32), hepatocellular (33), and other carcinomas, and therefore may be more prevalent than previously thought. For example, in a recent study of primary tumor cells from MM patients, Markovina et al. (30) found that all patients’ cells constitutively expressed NF-κB activity, and that bortezomib effectively blocked proteasome activity in all. However, in 10 of 14 cases, bortezomib failed to inhibit NF-κB activity, and in 8 of these cases bortezomib augmented NF-κB activity. Thus, the combination of bortezomib with an agent such as milatuzumab, which can independently down-regulate NF-κB, may be especially relevant.

Bortezomib may cause cell death through additional pathways not dependent on NF-κB. Thus, various scenarios can be envisioned to explain the enhancement of cytotoxicity by the combination of milatuzumab and bortezomib. If the tumor has constitutive NF-κB that is resistant to bortezomib, then bortezomib toxicity will only be due to apoptotic effects that are independent of NF-κB activation (32, 33). Down-regulation of NF-κB by milatuzumab will add to the toxicity by introducing a second mechanism of killing. Alternatively, if the tumor is one in which bortezomib does overcome NF-κB activation, then the combination of milatuzumab and bortezomib would be expected to yield greater decreases in NF-κB activity, again leading to greater cell killing. Because myeloma cell lines differ in baseline signaling characteristics (34), CD74 expression, and activation of NF-κB response to bortezomib, a more detailed evaluation of concentration and time effects on a panel of cell lines will be necessary to fully characterize CD74 and bortezomib interactions at the subcellular level.

Although our in vitro studies showed the sensitivity of CD74+ MM cell lines to doxorubicin, no significant effect was observed when doxorubicin was used as a single agent in CAG-bearing SCID mice. Milatuzumab monotherapy was markedly more effective than doxorubicin, and the doxorubicin + milatuzumab treatment protocol administered did not significantly improve the efficacy over that of milatuzumab alone. However, it should be noted that other protocols of doxorubicin administration, such as fractionated or multiple dosing, have not yet been evaluated in this model, and may yield some efficacy in vivo. Moreover, in light of results of a phase III study in relapsed or refractory MM, which showed that the combination of pegylated liposomal doxorubicin plus bortezomib is superior to bortezomib monotherapy (35), combinations of milatuzumab with bortezomib plus doxorubicin will be tested.

In conclusion, the function of CD74 as a survival receptor, combined with the expression of CD74 on malignant B cells and limited expression on normal tissues, implicate CD74 as a target for MM therapy. Moreover, the therapeutic efficacies of bortezomib, doxorubicin, and dexamethasone are enhanced when given in combination with milatuzumab. Although gaps remain in our understanding of the mechanisms of cytotoxicity of milatuzumab, it seems likely that interference with the survival functions of CD74 is involved. Future studies will focus on this issue as well as gaining a greater understanding of milatuzumab and drug interactions at the subcellular level, to aid in the rational design of combined modality regimens.

Disclosure of Potential Conflicts of Interest

D.M. Goldenberg declares financial interest (officer, stock, patents) in Immunomedics, Inc. The other authors declare no financial conflict.

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Combining Milatuzumab with Bortezomib, Doxorubicin, or Dexamethasone Improves Responses in Multiple Myeloma Cell Lines

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