Mcl-1 Is a Relevant Therapeutic Target in Acute and Chronic Lymphoid Malignancies: Down-Regulation Enhances Rituximab-Mediated Apoptosis and Complement-Dependent Cytotoxicity


Abstract

Purpose: The antiapoptotic Bcl-2 family member protein Mcl-1 is dynamically regulated in transformed B-cells, has a short mRNA and protein half-life, and is rapidly processed during apoptosis. Multiple therapies cause down-regulation of Mcl-1 in chronic and acute lymphoid leukemia (CLL and ALL) cells. Mcl-1 has also been reported to mediate resistance to rituximab in CLL. We therefore investigated whether direct reduction of Mcl-1 was sufficient to induce apoptosis and increase sensitivity to rituximab.

Experimental Design: We used Mcl-1–specific small interfering RNA in ALL cell lines and tumor cells from CLL patients to block transcription of Mcl-1.

Results: We show that Mcl-1 down-regulation alone is sufficient to promote mitochondrial membrane depolarization and apoptosis in ALL and CLL cells. Given the importance of rituximab in B-cell malignancies, we next assessed the influence of Mcl-1 down-regulation on antibody-mediated killing. Mcl-1 down-regulation by small interfering RNA increased sensitivity to rituximab-mediated killing both by direct apoptosis and complement-dependent cytotoxicity, but did not enhance antibody-dependent cellular cytotoxicity.

Conclusions: These results show that Mcl-1 is a relevant therapeutic target for ALL and CLL, and its down-regulation has the potential to enhance the therapeutic effect of rituximab in CD20-bearing lymphoid cells.

A key factor in inappropriate cell survival in acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) is the imbalanced expression of Bcl-2 family member proteins. Members of this family function both in proapoptotic and antiapoptotic capacities, and multiple studies link the antiapoptotic subset (Bcl-2, Bcl-xl, and Mcl-1) to drug resistance and poor treatment outcome in a variety of tumor types (1–9). Bcl-2, Bcl-xl, and Mcl-1 are localized predominantly in the outer mitochondrial membrane and function to preserve its integrity by binding to mitochondrial porin channels, thus inhibiting mitochondrial destabilization and subsequent initiation of apoptosis (5, 10, 11).

Mcl-1 also protects mitochondria by sequestering proapoptotic Bcl-2 family members, including BH3-domain–containing proteins as well as Bax and Bak. Reduction in Mcl-1 protein is thought to free these proapoptotic proteins to initiate mitochondrial membrane disruption and subsequent release of cytochrome c, which then activates the intrinsic pathway of apoptosis (11–13). Importantly, although Bcl-2 and Bcl-xl proteins both have relatively long half-lives, the estimated half-life of Mcl-1 in U937 cells is ~3 h (14). Finally, Mcl-1 expression is rapidly induced by B-cell receptor ligation (15), CD40 (3), and vascular endothelial growth factor (16, 17), providing evidence that it is strongly associated with B-cell survival. Together, these findings substantiate Mcl-1 as an intriguing therapeutic target.

In some cell lines (17, 18), down-regulation of Mcl-1 by small interfering RNA (siRNA) or Mcl-1 antisense molecules is sufficient to initiate apoptosis. In other cell types, however, down-regulation of Mcl-1 is insufficient to initiate apoptosis but promotes sensitivity to radiation and chemotherapy (19–21). To date, the therapeutic benefit of Mcl-1 down-regulation in lymphoid malignancies has not been adequately explored, and the influence of this important survival protein on protection of cells from antibody-mediated killing is unknown.

The therapeutic antibody rituximab represents a major advance in the treatment of CD20-positive B-cell malignancies. This anti-CD20 antibody mediates its cytotoxic effect through direct antibody-mediated apoptosis, antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC). Processing of caspase-3 and caspase-9, but not caspase-8, occurs in CLL patients who respond to rituximab,
suggesting activation of the intrinsic pathway of apoptosis (22). The potential importance of Mcl-1 in rituximab resistance was supported by results from a single-agent clinical trial in relapsed CLL, in which samples from patients who did not respond to rituximab showed a significantly higher Mcl-1/Bax expression ratio than samples from patients who responded to treatment (23). The objective of this work was to determine whether down-regulation of Mcl-1 plays a primary role in the initiation of apoptosis in B-cell leukemia, providing justification for the development of Mcl-1–targeted therapies. Furthermore, we sought to determine if direct Mcl-1 down-regulation enhances the therapeutic benefit of monoclonal antibodies such as rituximab. Our results indicate that Mcl-1 reduction by itself is sufficient to induce apoptosis in ALL and CLL cells, and that this also enhances the therapeutic benefit of rituximab.

**Materials and Methods**

*Patient samples and cell culture.* Written, informed consent was obtained to procure blood samples from CLL patients according to the Declaration of Helsinki and approved by The Ohio State University Institutional Review Board. CLL cells from 17 patients were used for Mcl-1 siRNA transfection and subsequent cytoxicity studies. Patient demographics are shown in Table 1. Patients were untreated for at least 6 weeks at the time of sample collection, and the median leukocyte counts of samples used for this study were >30,000 cells/μL. CD19-positive cells were isolated from peripheral blood using Rosette-Sep (StemCell Technologies, Vancouver, BC). Cells were incubated at 37°C, 5% CO2 in Hybirdoma SFM (Invitrogen, Carlsbad CA) supplemented with l-glutamine, penicillin, and streptomycin (Sigma, St. Louis MO). The 697 and RS4;11 ALL cell lines were obtained from DSMZ (Braunschweig, Germany). The Raji cell line was obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI 1640 with 10% fetal bovine serum plus supplements as above. Z-VAD-fmk (Enzyme Systems Products, Livermore, CA) was used at 150 μmol/L. Rituximab and the control anti–HER-2/Neu monoclonal antibody herceptin (Genentech, San Francisco, CA) were obtained commercially. Goat anti-human IgG antibody (Fc χ fragment-specific) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Transfection.** 697, RS4;11, and Raji cells were passed 24 to 36 h before transfection. Cells (3.0 × 10^6) were resuspended in Nucleofector solution (Nucleofector kit R, Amaza Biosystems, Cologne, Germany) with 1.45 μg of nonsense siRNA or Mcl-1 siRNA (HP GenomeWide siRNA, Qagen, Valencia CA) or Mcl-1 siRNA (Silencer Pre-designed siRNA, Ambion, Austin TX) using the Amaza Nucleofector apparatus and programs T-16 or L-17. Our results (i.e., reduction of Mcl-1 protein relative to nonsense control) in each cell type confirmed that both products gave similar percentage decrease in Mcl-1 protein relative to the nonsense control, although program L-17 gave slightly lower background cell death and was selected for the remaining experiments. Immediately after transfection, cells were transferred into RPMI 1640 with 10% fetal bovine serum. CLL cells (7 × 10^6) were resuspended in Nucleofector solution (Nucleofector kit V) with 2.7 μg nonsense or Mcl-1 siRNA (Nucleofector program U-16). Immediately after transfection, cells were transferred to Hybirdoma SFM. Nonsense siRNA and/or pGFP (green fluorescent protein), included in Amaza kit, served as negative controls. Based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assays determining the time course for induction of cell death, cells were collected 24 and 44 h posttransfection for apoptosis and Mcl-1 expression studies.

**Apoptosis and flow cytometric studies.** Apoptosis was assessed by flow cytometry using Annexin V-FITC and/or propidium iodide (BD Biosciences, San Diego, CA). Mitochondrial membrane potential changes were assessed using the lipophilic cationic dye JC-1 (Molecular Probes, Eugene, OR). Results (percentage of negative cells) were normalized to either untransfected cells or cells transfected with the nonsense siRNA control. Mock-transfected cells (transfected with no siRNA) produced results similar to nonsense siRNA transfection. To assess direct cytoxicity with rituximab and the control antibody herceptin against Raji and CLL cells transfected with Mcl-1 siRNA, we performed Annexin V-FITC and propidium iodide flow cytometry to determine the percentage of live cells.

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**Table 1.** Characteristics of CLL patient samples

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<th>Prior treatment</th>
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Abbreviation: NA, not analyzed.

*Progressive disease is defined as progression to treatment within 6 mo.

†Relative percentage of live (Annexin and propidium iodide negative) Mcl-1 siRNA-transfected cells, compared with cells transfected with nonsense siRNA.

All patients had interphase cytogenetic evaluation of at least del(13q14), del(11q22.3), del(17p13.1), and trisomy 12 (+12) by fluorescence in situ hybridization.

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ADCC and CDC assay. ADCC activity was determined by standard 4-h 51Cr-release assay. 51Cr-labeled target cells (1 × 10⁶ Raji cells per well) were placed in 96-well plates, and various concentrations of antibodies were added. Mononuclear cell isolates from healthy donors were then added to the plates at indicated effector to target ratios. After a 4-h incubation, supernatants were counted in a gamma counter. The percentage of specific cell lysis was calculated as percentage lysis = 100(ER/SR) / (MR – SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release, respectively. For the CDC assay, Raji cells at 1.0 × 10⁶/mL were suspended in RPMI medium with 1% normal human serum with or without heat inactivation (56°C, 30 min). The percentage of serum required was determined by titration of 1% to 30% untreated serum with rituximab on Raji cells. One percent was selected as the level that, with rituximab, produced ~50% relative cell death by propidium iodide positivity in the conditions of the assay (data not shown). Rituximab or herceptin was then added to 10 µg/mL. After 1 h incubation at 37°C, cells were analyzed by propidium iodide flow cytometry. The extent of CDC was measured as percentage propidium iodide–positive cells in duplicate samples.

Immunoblot analysis. SDS-PAGE/immunoblotting was done using standard procedures. Antibodies used were anti-Mcl-1, anti-β-actin, and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz CA). Band intensity was measured digitally using a ChemiDoc apparatus (Bio-Rad, Hercules, CA).

Quantitative real-time PCR. Total RNA was isolated 24 and 44 h after transfection using the Purescript RNA Purification Cell and Tissue kit (Gentra Systems, Minneapolis, MN). Quantitative real-time PCR analysis for Mcl-1 (Genbank accession no. NM_021960) was done on an ABI Prism 7900 System using TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA). Samples were tested in triplicate and Mcl-1 mRNA levels were normalized to that of 18S rRNA (Applied Biosystems). ΔCt values were obtained by subtracting the cycle number at which Mcl-1 signal reached a specific level (Ct value) from the 18S RNA Ct value in the same sample.

Statistical analysis. The significance of differences between mock control and nonsense RNA or Mcl-1 siRNA was determined using the Student’s t test.

Results

Mcl-1 down-regulation promotes apoptosis in acute and CLL cells. The acute leukemia cell lines 697, RS4;11, and Raji were either untransfected or transfected with Mcl-1–specific siRNA or nonsense (scrambled siRNA) control. For each experiment, cells were also transfected with no siRNA (mock transfected). Data are shown for 697 cell experiments. The same experiments were done using RS4;11 and Raji cells, and in each case similar results were obtained. We first examined the effect of Mcl-1 siRNA transfection on cell death over time. Cells transfected with the Mcl-1–specific siRNA had decreased viability by 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay compared with the nonsense controls that was significant by 22 h (Fig. 1A). This cytotoxicity did not further diminish relative to the nonsense-transfected cells, which could reflect either a partial recovery of mitochondrial health in transfected cells or new cell proliferation. Similar but enhanced effects were observed using Annexin/propidium iodide flow cytometry (Fig. 1B). We therefore selected the 22 to 24 h time point for further experiments.

We then examined Mcl-1 protein expression in siRNA-transfected 697 cells. Mcl-1 siRNA produced an ~67% reduction of Mcl-1 mRNA relative to the nonsense control (Fig. 2A) that paralleled the loss in cell viability. This was accompanied by reduction in Mcl-1 protein levels (Fig. 2B). As Mcl-1 is a target of degradation by caspase-3 (24), we sought to determine if reduction in Mcl-1 mRNA and protein was due to the siRNA or to an indirect effect of caspase activation induced by the transfection protocol. The pan-caspase inhibitor Z-VAD-fmk reduced apoptotic cell death as detected by Annexin/propidium iodide staining (Fig. 2C), but siRNA-mediated reduction of Mcl-1 protein was not affected (Fig. 2B).

Mcl-1 has been implicated in the stabilization of the mitochondrial membrane via its binding and sequestering of proapoptotic Bcl-2 family members as well as binding to mitochondrial membrane channels. Therefore, we investigated the effect of Mcl-1 down-regulation on the mitochondrial membrane potential. To do this, we used the lipophilic dye JC-1, which forms red fluorescent aggregates in polarized mitochondria. As membrane potential dissipates, JC-1 disperses to green-fluorescing monomers and this transition is detectable...
using flow cytometry (25, 26). As shown in Fig. 2D, Mcl-1 siRNA induced a notable mitochondrial membrane depolarization in 697 cells relative to the nonsense control, indicated by arrows in the figure. This loss of JC-1 aggregates was not prevented by preincubation with Z-VAD-fmk. These results suggest that Mcl-1 functions to prevent the mitochondria from undergoing depolarization, thereby preventing apoptosis via the mitochondrial pathway.

We then did similar experiments using CD19-positive B cells isolated from CLL patients. Figure 3A shows that CLL patient cells had a significant increase in cell death at 24 h after transfection with Mcl-1–specific siRNA relative to nonsense control siRNA. This cytotoxicity was accompanied by loss of Mcl-1 mRNA (Fig. 3B) and protein (Fig. 3C). As in 697 cells, cell death induced by Mcl-1 siRNA was blocked by Z-VAD-fmk (Fig. 3A), but loss of mitochondrial membrane potential (Fig. 3D) as well as down-regulation of Mcl-1 protein (Fig. 3C) was not inhibited. Interestingly, Mcl-1 siRNA-induced apoptosis was unrelated to the stage of disease or other clinical variables of patients from whom samples were obtained (Table 1), supporting the relevance of Mcl-1 as a therapeutic target even in drug-resistant CLL.

Mcl-1 down-regulation enhances rituximab-mediated apoptosis and CDC, but not ADCC. Given the importance of both the intrinsic apoptotic pathway in rituximab-mediated cytoxicity, as well as the clinical demonstration that pretreatment Mcl-1/ Bax ratio correlates with poor response to rituximab, we examined whether specific down-regulation of Mcl-1 enhanced the ability of rituximab to mediate cell killing. We used Raji cells for this purpose, as they express CD20 and are an accepted model to investigate antibody-mediated killing in lymphoma. Furthermore, rituximab has known clinical activity against Burkitt lymphoma (27), and rituximab has cytoxicity in both lymphoid cell lines and CLL patient cells (28). As was observed with 697 cells, Mcl-1–specific siRNA induced substantial apoptosis in Raji relative to untransfected and nonsense siRNA

![Fig. 2.](image-url)
controls (Fig. 4A), and also caused a notable reduction in Mcl-1 protein expression (Fig. 4B). Mcl-1--specific siRNA, but not the nonsense control, enhanced direct apoptosis mediated by rituximab concurrent with Mcl-1 down-regulation (Fig. 4C). A similar effect on cytotoxicity was observed with CLL cells (Fig. 4D). In contrast, there was not a significant effect with the control monoclonal antibody herceptin. We then examined additional mechanisms by which rituximab induces cell death. We observed a moderate increase in CDC when Raji cells treated with Mcl-1 siRNA were incubated with rituximab together with untreated human serum. When heat-fixed serum from the same source was used, no additional cytotoxicity was detected with rituximab relative to the controls (Fig. 4E). Interestingly, although ADCC is a mechanism of rituximab-mediated cell killing (29, 30), enhancement of rituximab-mediated ADCC was not observed after treatment with Mcl-1 siRNA (data not shown). Overall, these data indicate that down-regulation of Mcl-1 influences some but not all death pathways activated by rituximab, and that strategies targeting Mcl-1 expression may enhance the efficacy of rituximab therapy.

Figure 3. Mcl-1 gene silencing in CLL patient cells: CD19-positive cells from CLL patients were untransfected, mock-transfected, or transfected with nonsense or Mcl-1 siRNA. A, percentage live (Annexin and propidium iodide negative) CLL cells 24 h posttransfection. CLL cell death is significantly increased relative to the nonsense control at 24 h (*, P = 0.01; n = 7), and this effect is abrogated by the broad caspase inhibitor Z-VAD-fmk. B, Mcl-1 mRNA is reduced relative to 18S RNA in siRNA-treated CLL cells. Cells from CLL patients (n = 3) were analyzed for Mcl-1 expression by real-time reverse transcription-PCR 24 h posttransfection. C, protein expression of Mcl-1 after transfection with Mcl-1 siRNA with or without Z-VAD-fmk. Numbers (bottom), Mcl-1 signal relative to the actin control. The immunoblot is representative of three experiments. D, loss of mitochondrial membrane potential in Mcl-1 siRNA-treated CLL cells. Values shown in this experiment were calculated relative to the nonsense siRNA-transfected cells, set at 100%. Data are representative of three experiments.

Discussion

In this work, we show that reduction of Mcl-1 expression alone is sufficient to activate apoptosis in ALL and CLL using both transformed cell lines and primary tumor cells from CLL patients. Like many other survival proteins, Mcl-1 is rapidly degraded and inactivated by activated caspases during apoptosis, making changes in this protein in the context of drug or even siRNA therapy difficult to interpret. To show the specificity for our siRNA down-regulation of Mcl-1, we inhibited caspase activation and yet still observed down-regulation of Mcl-1 mRNA and protein levels concurrent with depolarization of the mitochondrial membrane. These data suggest that Mcl-1 down-regulation by siRNA is a primary event in initiating apoptosis. Furthermore, induction of apoptosis by down-regulation of Mcl-1 seems to be a general phenomenon that is not affected by disease stage, progression, or chromosomal deletions. Our data also support the hypothesis that Mcl-1, which provides resistance to apoptosis in CLL as well as other malignancies, is a particularly important factor in this pathway and is not simply a bystander in the complex apoptotic process. As Mcl-1 is an important survival factor in lymphoid malignancies, these data provide significant input for drug development in B-cell acute and CLL drug development. This report represents what we believe to be the first effective use of siRNA to validate a potential drug target in ALL and primary CLL cells. For the many therapies such as flavopiridol (31), roscovitine (32), and BAY 43-9006 (33, 34), with which significant down-regulation of
Mcl-1 mRNA and/or protein is noted, our data support Mcl-1 as a therapeutic target. Additionally, Balakrishnan et al. (35) found that 8-amino-adenosine, an RNA-directed nucleoside analogue, caused reduction of antiapoptotic proteins, including Mcl-1, leading to cell death. Our data also suggest that therapies that specifically target Mcl-1, such as micro-RNA, could be effective in the treatment of ALL and CLL and should be further investigated. In this regard, Mcl-1–specific antisense oligonucleotides

Fig. 4. Mcl-1 down-regulation enhances cytotoxicity of rituximab. A, Raji cells were either untransfected or transfected with nonsense or Mcl-1 siRNA. The percentage of Annexin- and propidium iodide–negative cells 24 h posttransfection with Mcl-1 siRNA was significantly decreased relative to the nonsense control (P = 0.003). Data are shown relative to untransfected cells, set at 100%. B, Mcl-1 protein expression in Mcl-1 siRNA transfected Raji cells 24 h posttransfection by immunoblot. Numbers (bottom), Mcl-1 signal relative to the tubulin control. C, direct cytotoxicity assay using Raji cells untransfected or transfected with nonsense or Mcl-1 siRNA. At 22 h, cells were incubated with goat anti-human IgG antibody cross-linker alone, cross-linker plus rituximab, or cross-linker plus herceptin. After 24 h, apoptosis was analyzed by Annexin/propidium iodide staining (n = 3; P = 0.003). D, direct cytotoxicity assay using CLL cells untransfected or transfected with nonsense or Mcl-1 siRNA. After 22 h, cells were incubated with cross-linker alone, cross-linker plus rituximab, or cross-linker plus herceptin. After 24 h, apoptosis was assessed by Annexin/propidium iodide staining (n = 8; P = 0.008). E, effect of Mcl-1 down-regulation on rituximab–mediated complement-dependent cytotoxicity. Raji cells were incubated in medium with 1% untreated or heat-inactivated serum, treated with antibodies, and incubated for 1 h. The extent of lysis was measured by flow cytometry analysis of propidium iodide fluorescence (n = 5; P = 0.042).
have been successfully used in solid tumor xenograft models, further supporting Mcl-1 as a viable therapeutic target in cancer (21, 36). Of greater interest is our confirmation that Mcl-1 expression contributes to rituximab-mediated resistance in lymphoid malignancies. Previously, we showed that elevated Mcl-1/Bax ratios pretreatment predicted resistance to rituximab-mediated therapy in CLL (23). Rituximab works through several mechanisms, including direct induction of apoptosis, CDC, and ADCC. We sought to study the influence of Mcl-1 downregulation using the lymphoblastic cell line Raji, in which all three mechanisms of antibody-mediated killing can be effectively studied. We observed that Raji cells transfected with Mcl-1 siRNA had 20% greater apoptotic cell death compared with the nonsense control after treatment with rituximab (Fig. 4C). In Mcl-1 siRNA-transfected CLL cells, sensitivity to rituximab was enhanced by an average of 17%. However, there was greater variability between CLL patient samples than in the Raji cell line, with two patient samples showing >25% cell death in Mcl-1–transfected cells relative to the rituximab-treated nonsense control (Fig. 4D). Similarly, rituximab-mediated CDC was modestly enhanced in Raji cells with siRNA downmodulation of Mcl-1 (Fig. 4E). These data provide a rationale for combining rituximab therapy with agents that directly down-regulate Mcl-1 protein expression in lymphoid malignancies, particularly in diseases such as CLL in which apoptosis, and likely complement, contribute to the mechanism of tumor elimination in vivo.

References


26. Salvio S, Angelini A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess i.e., changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.


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