A 13 mer LNA-i-miR-221 Inhibitor Restores Drug Sensitivity in Melphalan-Refractory Multiple Myeloma Cells

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Abstract

Purpose: The onset of drug resistance is a major cause of treatment failure in multiple myeloma. Although increasing evidence is defining the role of miRNAs in mediating drug resistance, their potential activity as drug-sensitizing agents has not yet been investigated in multiple myeloma.

Experimental Design: Here we studied the potential utility of miR-221/222 inhibition in sensitizing refractory multiple myeloma cells to melphalan.

Results: miR-221/222 expression inversely correlated with melphalan sensitivity of multiple myeloma cells. Inhibition of miR-221/222 overcame melphalan resistance and triggered apoptosis of multiple myeloma cells in vitro, in the presence or absence of human bone marrow (BM) stromal cells. Decreased multiple myeloma cell growth induced by inhibition of miR-221/222 plus melphalan was associated with a marked upregulation of pro-apoptotic BBC3/PUMA protein, a miR-221/222 target, as well as with modulation of drug influx-efflux transporters SLC7A5/LAT1 and the ABC transporter ABCC1/MPR1. Finally, in vivo treatment of SCID/NOD mice bearing human melphalan-refractory multiple myeloma xenografts with systemic locked nucleic acid (LNA) inhibitors of miR-221 (LNA-i-miR-221) plus melphalan overcame drug resistance, evidenced by growth inhibition with significant antitumor effects together with modulation of PUMA and ABCC1 in tumors retrieved from treated mice.

Conclusions: Taken together, our findings provide the proof of concept that LNA-i-miR-221 can reverse melphalan resistance in preclinical models of multiple myeloma, providing the framework for clinical trials to overcome drug resistance, and improve patient outcome in multiple myeloma. Clin Cancer Res; 22(5): 1222–33. ©2015 AACR.

Introduction

Multiple myeloma is characterized by the abnormal proliferation of malignant plasma cells in the bone marrow (BM; refs.1, 2). Despite recent advances in multiple myeloma biology (3), preclinical models (4–6), and translation of novel agents which have markedly improved the outcome of multiple myeloma patients, the development of drug resistance remains an obstacle to long-term survival (7). Multiple myeloma commonly progresses to drug-refractory end-stage disease (8), and novel therapeutic strategies are urgently needed.

For more than 30 years, melphalan has been the mainstay of multiple myeloma treatment (9). Presently its therapeutic value is in younger patients who undergo high-dose melphalan prior to autologous stem cell transplantation, as well as in nontransplant candidates or elderly patients as a part of first-line combination regimens (10, 11). The use of melphalan combination regimens with new agents, such as bortezomib or lenalidomide, has significantly prolonged progression-free survival and overall survival; however, development of drug resistance leads to relapse of disease (12). Recently, a renewed scientific interest on melphalan is emerging, and major efforts have been devoted to delineate the mechanisms underlying primary or acquired melphalan resistance (13). These efforts have already led to the design of novel regimens to overcome melphalan resistance or to improve its antitumor activity (14).

Currently, there is a growing interest for the therapeutic potential of strategies aimed to target miRNAs network. miRNAs are a class of short ncRNA that function as post-transcriptional gene regulators. miRNAs mainly act through complete or partial binding to 3′-UTR of their mRNA targets, inducing either mRNA degradation or translational repression (15). By targeting driver genes involved in critical cellular pathways, miRNAs can function as oncogenes or tumor suppressor genes, playing a key role in tumorigenesis, as well as in cancer progression and aggressiveness.

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**Translational Relevance**

Dysregulation of oncogenic miRNAs is frequently involved in cancer progression up to drug-resistant stage. We investigated the role of miR-221/222 in melphalan resistance in multiple myeloma cells. The translational relevance of our study relies in the demonstration of in vivo efficacy of a novel 13-mer locked nucleic acid (LNA)-miR-221 inhibitor in restoring melphalan sensitivity of refractory multiple myeloma cells by selective interference with relevant molecular mechanisms of resistance. Importantly, LNA-i-miR-221 exerts anti-multiple myeloma activity by itself and is suitable for systemic delivery. Our findings provide therefore the rationale for clinical investigation of LNA-i-miR-221 plus melphalan in drug-refractory stage of disease.

(15). A variety of studies have, to date, demonstrated the potential relevance of miRNA mimics/inhibitors as therapeutic tools, and the promising results from the first phase-II trial in patients with HCV infection treated with locked nucleic acid (LNA)-miR-122 inhibitors have further stimulated studies for the treatment of human cancer (16). In multiple myeloma, miRNA-based strategies are presently emerging as promising approaches (17–25). Moreover, recent findings have emphasized the role of miRNAs in the development of drug resistance in a variety of malignancies (26). In particular, miRNAs have been shown to regulate drug efflux transporters, induction of apoptosis, cell-cycle progression, DNA repair mechanisms, and other alterations of drug targets (27). Among miRNAs involved in development of drug resistance, miR-221/222 plays a key role: inhibition of miR-221/222 has been reported to overcome resistance to cisplatin (28), tamoxifen (29), fulvestrant (30), temozolamide (31), TKIs (32), and TRAIL (33) in a variety of cancers. We recently provided the first evidence that silencing of miR-221/222 by specific inhibitors exerts antitumor activity in multiple myeloma cells bearing t(4;14) translocations in vitro and in vivo (34), and that naked LNA inhibitors of miR-221 (LNA-miR-221) are suitable for systemic delivery in animals (35). Here we investigated the role of miR-221/222 in melphalan-refractory multiple myeloma, and demonstrate restoration of melphalan sensitivity in refractory cells after exposure of multiple myeloma cells to a novel 13-mer LNA-i-miR-221. Our findings, therefore, provide the rationale for clinical trials investigating LNA-i-miR-221 plus melphalan in drug-refractory multiple myeloma.

**Materials and Methods**

**Cell cultures, reagents, and drugs**

Multiple myeloma cell lines NCI-H929 t(4;14), RPMI-8226 t(14;16) and U266 t(11;14) were purchased from DSMZ (Germany) which certified authentication performed by STRs DNA typing. These cells were immediately frozen and used from the original stock within 6 months. Melphalan-resistant U266/LR7 t(11;14) cells were kindly provided by Dr. A. Pandiella (Instituto de Biología Molecular y Celular del Cáncer, CSIC-Universidad de Salamanca, Salamanca, Spain). AMO1 t(12;14) and bortezomib-resistant AMO1 Abz (12;14) cells were kindly provided by Dr. C. Driessen (Eberhard Karls University, Tübingen, Germany), U266/LR7, AMO1, and AMO1 Abzb were not further authenticated but confirmed for the described drug-resistant phenotype. All cells were cultured in RPMI-1640 (Gibco, Life Technologies), as previously described (36, 37). Human stromal HS-5 cells were purchased from ATCC, which certify authentication by STRs profiling. Also, these cells were immediately frozen and used from the original stock within 6 months. HS-5 were cultured in DMEM (Gibco, Life Technologies) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Following informed consent and Institutional Ethical Committee’s approval, PBMCs and primary CD138+ multiple myeloma cells from BM aspirates of 3 patients with multiple myeloma were isolated, as previously described (38). LNA-i-miR-221 was designed and synthesized as previously described (35). Melphalan and bortezomib were purchased from Sigma–Aldrich and Selleck Chemicals, respectively.

**In vitro transfection of multiple myeloma cells**

Synthetic mirVana miR-221 and miR-222 inhibitors or mimics were purchased (Life Technologies); mirVana miRNA mimic and inhibitor Negative Control #1 (Life Technologies) were used as experimental negative controls (NC). A total of 1 × 10⁵ multiple myeloma cells were transfected at 100 nmol/L miRNAs concentrations by the Neon Transfection System (Life Technologies; 1050V, 2 pulse, 30 A); transfection efficiency, evaluated by flow-cytometry analysis relative to a FAM dye–labeled anti-miR-negative control, reached 85% to 90%. Similar conditions were applied for transfection of multiple myeloma cells with Silencer Select siRNA for PUMA/BBC3 (siPUMA) or with Silencer Select siRNA control (siCNT) [Life Technologies], which was used at final concentration of 50 nmol/L even in cotransfection experiments with miRNA inhibitors.

**Virus generation and infection of human stromal HS-5 cells**

HS-5 cells stably expressing GFP transgene were obtained, as previously described (39; see Supplementary Methods for detailed information).

**Reverse transcription and quantitative real-time PCR**

Total RNA extraction from multiple myeloma cells and qRT-PCR were performed as previously described (see Supplementary Methods for detailed information; ref. 38).

**Cell proliferation and survival assay**

Cell growth inhibition was evaluated by Cell Counting Kit-8 (CCK-8) colorimetric assay (Dojindo Molecular Technologies, Inc.), according to the manufacturer’s instructions. For melphalan dose–response experiments, multiple myeloma cells were seeded in 24-well plates at a density of 2.5 × 10⁵ cells per well in 1 mL of culture medium and incubated for 24 hours in the presence of different μmol/L melphalan concentrations; after incubation, multiple myeloma cells were inoculated in 96-well plates for CCK-8 assay. Final optical density (O.D.) was measured at 450 nm using GloMax (Promega). Wells without cells (culture medium alone) were used as blank. For combination experiments with miRNAs, 1 × 10⁵ electroporated cells with NC or miR-221/222 inhibitors were incubated for 24 hours in 6-well plates; after harvesting, cells were seeded in 24-well plates at a density of 2.5 × 10⁵ cells/mL and incubated in the presence or absence of different μmol/L melphalan concentrations. Twenty-four hours after beginning drug exposure, cells were seeded in 96-well plates for CCK-8 assay and O.D. measurement. For coculture experiments, 2.5 × 10⁵ U266/LR7 cells transfected with
miR-221/222 inhibitors or NC were adhered to a monolayer of GFP + HS-5 cells at 50% confluence in 12-well plates for 24 hours. After incubation, cocultured cells were treated with 100 μmol/L of melphalan, and collected 24 hours later by gentle pipetting for 7-aminoglutethimide D (7-AAD) staining and flow cytometry analysis. Each experiment was repeated at least three times. Data represent the mean ± SD of at least three independent experiments.

**Apoptosis detection by fluorescence microscopy**

A total of 5 × 10^3 U266/LR7 cells transfected with NC or miR-221/222 inhibitors were incubated for 24 hours in 12-well plates; after harvesting, each group was incubated in the presence or absence of 100 μmol/L melphalan. Twenty-four hours later, harvested cells were plated in 96-well plates and visualized directly in the culture dishes after staining with Hoechst 33342 and Propidium Iodide (PI). Briefly, treated cells were washed and suspended in PBS × 1 at a density of 1 × 10^3/mL with the addition of 5 μmol/L Hoechst 33342 (Sigma–Aldrich). After incubation at 37°C for 15 minutes, cells were washed and resuspended in Binding Buffer × 1 (BD Pharmingen) added with 0.5 μmol/L PI (BD Pharmingen), and incubated at room temperature for 15 minutes avoiding light exposure. After incubation, cells were washed and resuspended in PBS for fluorescence microscopy analysis using EVOS FL Auto Cell Imaging System (Life Technology). Representative fields of cells were visualized with the addition of Halt Protease Inhibitor (10 mmol/L Tris by electrophoresis on NuPAGE precasted gels (4% analysis, 50 μg beads (Santa Cruz Biotechnology) overnight at 4°C). The precipitates were resuspended in loading buffer and resolved by NuPAGE electrophoresis, followed by immune blotting after electrotransfer.

**Luciferase reporter experiments**

Validation of miR-221/222 direct targeting of PLM/A BBC3 3′-UTR was performed as previously described (see Supplementary Methods for detailed information; ref. 38).

**Protein extraction and Western blot analysis**

Total proteins were extracted with NP40 Cell Lysis Buffer (10 mmol/L Tris—HCl, pH 7.5, 150 mmol/L NaCl, 1% NP-40; Life Technologies) with the addition of Halt Protease Inhibitor Single-Use Cocktail × 100 (Thermo Scientific). For Western blot analysis, 50 μg per line of the whole lysates were separated by electrophoresis on NuPAGE precasted gels (4%–12%; Invitrogen, Life Technologies) and electro-transferred to nitrocellulose membrane (Trans-Blot Turbo Mini Nitrocellulose Transfer packs, Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad). After protein transfer, the membranes were blotted with the primary antibodies (see Supplementary Methods for detailed information).

**Immune precipitation**

Lysates were prepared by homogenizing retrieved tumor xenografts in NP-40 lysis buffer containing protease inhibitor by using gentleMACS Dissociator (Miltenyi Biotec). Immune precipitation was performed by incubating 1 mg of lysates with 2 μg of antibody and 30 μl of protein A/G-conjugated agarose beads (Santa Cruz Biotechnology) overnight at 4°C. The precipitates were resuspended in loading buffer and resolved by NuPAGE electrophoresis, followed by immune blotting after electrotransfer.

**Hoechst dye exclusion assay**

U266/LR7 cells were collected 48 hours after transfection with miR-221/222 inhibitors or NC and resuspended in PBS (× 1) at 1 × 10^3/mL concentration. Cells were pre-incubated at 37°C for 10 minutes in the presence or absence of verapamil 100 μmol/L (Sigma–Aldrich) to inhibit ABC transporters and then were incubated for 90 minutes at 37°C with Hoechst 33342 5 μg/mL (Sigma–Aldrich), with intermittent shaking. Subsequently, cells were washed and re-suspended in ice-cold PBS (× 1). 7-AAD was added (5 μL) to each tube prior to acquisition to exclude dead cells from the analysis. The cells were filtered to obtain single cell suspension for flow cytometry analysis performed by FACSAria III (Becton Dickinson). The Hoechst 33342 dye was excited at 357 nm, and its fluorescence was dual-wavelength analyzed (402–446 nm for Hoechst 33342-Blue; 650–670 nm for Hoechst 33342-Red).

**Animals and in vivo model of human multiple myeloma**

Six- to eight-week-old female SCID/NOD mice (Harlan laboratories, Inc.) were housed and monitored in Animal Research Facility at Magna Gracia University. All experimental procedures and protocols had been approved by the Institutional Ethical Committee (Magna Gracia University). In accordance with the institutional guidelines, mice were killed when their tumors reached 2 cm in diameter, or in the event of paralysis or major compromise in their quality of life. Specifically, we evaluated the in vivo ability of LNA-i-miR-221 (35), specifically designed for systemic delivery, to enhance the anti-multiple myeloma activity of melphalan. Briefly, a cohort of 24 mice for each in vivo study were subcutaneously inoculated with 5 × 10^5 U266/LR7 cells in 100 μL of RPMI-1640 medium; treatments started when tumors became measurable, approximately 3 weeks after cells were injected. In a first in vivo experiment, mice were randomized to receive four different treatments: (i) LNA-i-miR-221 intraperitoneally (i.p.) injected at a dose of 25 mg/kg at days 1, 4, 8, 15, and 22 from the beginning of the experiments; (ii) scrambled control with the same schedule; (iii) 0.75 mg/kg of i.p. melphalan twice weekly for 3 weeks; (iv) melphalan plus LNA-i-miR-221 with the above described schedules. In a subsequent in vivo study, treatment with LNA-i-miR-221 (25 mg/kg) plus melphalan (0.75 mg/kg) was given consecutively for 4 days every 10 days. Tumor sizes were measured as previously described (35). In vivo detection of the tumor mass in xenografted mice was performed using IVIS LUMINA II Imaging System (Caliper Life Sciences). At the end of the observation, tumors were retrieved from animals and placed in 10 formalin for histology or stored at −80°C for protein analysis.

**Histology and immunohistochemistry**

Retrieved tumors from animals were fixed in 4% buffered formaldehyde and 24 hours later washed, dehydrated, and embedded in paraffin. For light microscopy analysis by an optical microscope Nikon i55 (Nikon Corporation, Tokyo, Japan), we performed staining with H&E on 4-μm tumor sections mounted on poly-lysine slides. For IHC staining, 2-μm-thick tumor slices were de-paraffinized and pretreated with the Epitope Retrieval Solution 2 (EDTA buffer, pH 8.8) at 98°C for 20 minutes. After washing steps, peroxidase blocking was carried out for 10 minutes using the Bond Polymer. All procedures were performed using the Bond Max Automated Immunohistochemistry. Tissues were washed and incubated with the primary antibody directed against Ki-67 (Dako, clone MIB-1; 1:150) or caspase-3 (Novocastra, clone HM62; 1:500). Subsequently, tissues were incubated with polymer for 10 minutes.
and developed with DAB-Chromogen for 10 minutes. Slides were counterstained with Hematoxylin.

**Statistical analysis**

Each *in vitro* experiment was carried out at least three times, and all values are reported as means ± SD. Comparisons between groups were made with the Student *t* test, whereas statistical significance of differences among multiple groups was determined by GraphPad software (www.graphpad.com). Differences were considered significant with *P* ≤ 0.05. The Synergistic Index (SI) was determined as previously described (40) with the following formula: SI = (effect induced by miR-221/222 inhibitors and melphalan in combination)/effect of melphalan. Interactions were considered synergistic when SI was >1.

**Results**

Melphalan-induced modulation of miR-221/222 in multiple myeloma cells

We first evaluated the melphalan-sensitivity of our multiple myeloma cell line panel, including U266/S, NCI-H929, AMO1, RPMI-8226, AMO1 Abzb, and U266/LR7. NCI-H929, AMO1, and U266/S were sensitive at low melphalan concentrations; RPMI-8226 and the bortezomib-resistant AMO1 Abzb showed resistance at doses up to 20 μmol/L, whereas the melphalan-
resistant U266/LR7 cells were resistant up to 200 µmol/L drug concentration (IC_{50} not found; Fig. 1A). In parallel, we measured the miR-221/222 melphalan-induced expression in multiple myeloma cells. As evaluated by qRT-PCR, miR-221/222 expression after drug exposure inversely correlated with melphalan sensitivity (Fig. 1B and C). In particular, melphalan-resistant U266/LR7 cells showed the highest miR-221/222 expression after drug exposure. Significant miR-221 upregulation was also observed in RPMI-8226 and AMO1 Abzb cells, which are moderately resistant to melphalan, whereas those cell lines sensitive to melphalan, including NCI-H929, U266/S, and AMO1 cells, did not show any change in miR-221/miR-222 expression following treatment. Moreover, qRT-PCR analysis of miR-221 and miR-222 premelphalan treatment in U266/LR7 showed a 1.5-fold increase for miR-221 (Supplementary Fig. S1A) and a 3.5-fold increase for miR-222 (Supplementary Fig. S1B) compared with the parental-sensitive counterpart U266/S (Supplementary Fig. S1). Altogether, these findings demonstrate an inverse correlation of drug-induced miR-221/222 expression with melphalan sensitivity of multiple myeloma cells.

miR-221/222 inhibition overcomes melphalan resistance of multiple myeloma cells in vitro

We previously reported a strong in vitro and in vivo antitumor activity of miR-221/222 inhibitors in multiple myeloma cells bearing t(4;14) translocation (34). Since modulation of miR-221/222 seems to be affected by melphalan treatment independently from chromosome translocation, we investigated whether miR-221/222 plays a mechanistic role in multiple myeloma cell melphalan sensitivity/resistance regardless translocation status. To this aim, we first transfected U266/LR7, RPMI-8226 and AMO1 Abzb melphan-resistant cells with miR-221/222 inhibitors or scramble controls, and then exposed cells to increasing concentrations of melphalan. As shown in Fig. 2A, miR-221/222 inhibitors synergistically enhanced the anti-multiple myeloma effects of melphalan.

**Figure 2.** Antiproliferative and apoptotic effects in multiple myeloma cells of miR-221/222 inhibitors combined with melphalan. A, CCK-8 cell proliferation assay in U266/LR7, RPMI-8226, and AMO1 Abzb transfected with 100 nmol/L of miR-221/222 inhibitors or scrambled control (NC) and then treated with increasing melphalan concentrations. Data shown are the average of three independent experiments, and error bars represent SD of three independent experiments. B, Annexin V/7-AAD fluorescence microscopy after Hoechst 33343 and PI staining of melphalan-treated U266/LR7 cells (top) and caspase-3 levels in RPMI-8226 cells (bottom). Cells lines were transfected with 100 nmol/L of miR-221/222 inhibitors or NC and then exposed to melphalan (100 µmol/L for resistant U266/LR7 cells and 5 µmol/L for RPMI-8226 cells). Loading control was performed using GAPDH or γ-tubulin. C, Western blot analysis of total and cleaved caspase (Casp)-8, -9, and -3, and cleaved PARP in U266/LR7 cells (top) and caspase-3 levels in RPMI-8226 cells (bottom). Cells lines were transfected with 100 nmol/L of miR-221/222 inhibitors or NC and then exposed to melphalan (100 µmol/L for resistant U266/LR7 cells and 5 µmol/L for RPMI-8226 cells). Loading control was performed using GAPDH or γ-tubulin.
activity of melphalan in a dose-dependent manner. At a fixed concentration, (100 nmol/L) of miR-221/222 inhibitors with increasing doses of melphalan, the SI was measured: the highest synergistic effect was reached with 100 nmol/L of melphanal (SI = 5.6) in U266/LR7 cells, and with 5 nmol/L of melphalan (SI = 4.12) in RPMI-8226 cells, and in AMO1 Abzb cells (SI = 1.36; Supplementary Table S1). Conversely, enforced expression of synthetic miR-221/222 mimics in melphanal-sensitive U266/S cells, which, previously, we reported to exert a growth promoting activity on multiple myeloma cells (34), resulted in a reduced susceptibility to the drug (Supplementary Fig. S2A). Moreover, we evaluated whether miR-221+/222– sensitizing effects to melphalan could be due to induction of apoptosis. As shown in Fig. 2B using Annexin V/7-AAD assay, we found that the combined treatment triggered greater apoptosis in all multiple myeloma cell lines than each single agent. By Western blotting analysis, we showed activation/cleavage of CASPASE-8, -9, and -3, as well as increased PARP cleavage, in U266/LR7 cells exposed to combination treatment (Fig. 2C, top). An increase of cleaved CASPASE-3 was also confirmed by Western blotting in RPMI-8226 cells (Fig. 2C, bottom panel). Conversely, enforced expression of synthetic miR-221/222 mimics in U266/S cells exposed to melphalan resulted in a decrease of cleaved CASPASE-3 (Supplementary Fig. S2B). Using Hoechst 33342 and PI staining, we showed induction of apopto-
sis in cells after combination treatment, with a significant increase in late apoptosis (Fig. 2D). Importantly, inhibition of miR-221/222 enhanced the cytotoxic effect of melphalan even on primary CD138⁺ cells from 3 multiple myeloma patients (Fig. 3A). No effects on cell viability were instead observed on PBMCs from three healthy donors (Fig. 3B). We next studied whether bone marrow stromal cells (BMSCs) protected against the synergistic combination therapy of miR-221/222 plus melphalan. By qRT-PCR, a slight increase of miR-221/222 expression (2- and 4-folds, respectively) was detected in U266/LR7 cells adherent to the HS-5 BMSCs, as compared with cells cultured alone (Supplementary Fig. S3). Importantly, anti-multiple myeloma activity of miR-221/222 inhibitors plus melphalan against U266/LR7 cells adherent to HS5 BMSCs was similar to that against nonadherent U266/LR7 cells (Fig. 3C). Taken together, these findings demonstrate that miR-221/222 inhibitors enhance the therapeutic activity of melphalan in drug-resistant cells by induction of apoptosis, even in the presence of BMSCs.

**Genome-wide expression patterns triggered by miR-221/222 inhibitors plus melphalan**

To analyze the transcriptome perturbations induced in multiple myeloma cells by the combination of miR-221/222 inhibitors plus melphalan treatment, we performed a whole gene expression analysis (GEP) by GeneChip Affymetrix arrays. Data are available through GEO accession number GSE66618. Since we demonstrated that inhibition of miR-221/222 restores sensitivity to melphalan in resistant multiple myeloma cells, we next examined whether this effect may be associated with changes in the expression of genes involved in the most common mechanisms of drug resistance. We performed a GEP on both untreated or drug-exposed melphalan-resistant U266/LR7 cells and melphalan-sensitive parental U266/S multiple myeloma cells. By hierarchical clustering analysis, data points were grouped based on overall similarity in gene expression patterns. Clustering analysis revealed a similar profile in melphalan-resistant U266/LR7 multiple myeloma cells treated with miR-221/222 inhibitors plus melphalan, as was observed in the sensitive parental cells U266/S cells treated with melphalan alone (data not shown). Accordingly, we next performed fold change analysis, which yielded a list of 1487 genes modulated after combination treatment in melphalan-resistant multiple myeloma cells. Notably, differently expressed genes involved in apoptosis (PLA2G4B, BBC3, BCL2L1), drug transport (ABCC1/MRP1, SLC7A8, SLC7A5), and DNA repair mechanisms (XRCC4, PRKDC, CNOT6L, DDB2) were found (Supplementary Fig. S4A). By Ingenuity Pathway Analysis (IPA) software, we evaluated the perturbation of biological response pathways and regulatory networks. After IPA annotation, a list of 739 genes was used for “core analysis” and identification of different canonical pathways with highly-significant perturbation scores, primarily in the miR-221/222 inhibitors plus melphalan-treated U266/LR7 cells (P < 0.05; Supplementary Fig. S4B).

**Figure 3.**

Effects of miR-221/222 inhibitors plus melphalan on primary cells and on multiple myeloma cell lines in the presence of BMSCs. A, CCK-8 cell proliferation assay in primary CD138⁺ multiple myeloma patients cells transfected with 100 nmol/L of miR-221/222 inhibitors or scrambled control (NC) and then treated with 100 µmol/L of melphalan. Data shown are the average of two independent experiments, and P-values were obtained using two-tailed test. (*, P < 0.05). B, CCK-8 cell proliferation assay in primary PBMCs transfected with 100 nmol/L of miR-221/222 inhibitors or scrambled control (NC) and then treated with increased melphalan concentrations. Data shown are the average of three independent experiments, and P values were obtained using two-tailed test. (*, P < 0.05). C, 7-AAD staining of U266/LR7 cells transfected with 100 nmol/L of miR-221/222 inhibitors or NC, and then cultured in the presence or absence of GFP + hBMSCs. Twenty four hours after coculture, cells were treated with 100 µmol/L of melphalan; 24 hours later, they were collected and stained for 7-AAD flow cytometry analysis. The percentage of 7-AAD positive cells is represented. Values represent the mean ± SD of three independent experiments. (*, P < 0.05).
revealed modulation of pathways: associated with alkylation agents-induced response ("NRF2-mediated oxidative stress response"); induced by micro-environmental stimuli ("HGF signaling," "chemokine signaling," "PTEN signaling," "EIF2 signaling," and "PI3K signaling in B lymphocytes"); related to DNA repair mechanisms ("cell cycle: G_{2}-M DNA damage checkpoint regulation" and "DNA DSB repair by nonhomologous end joining"); as well as "glucocorticoid receptor signaling". Based on the IPA annotation analysis, we also characterized genes involved in these signaling pathways (Supplementary Fig. S4C, inserted table). Interestingly, we detected downregulation of ABCC1, PIK3CB, XRCC4, PRKDC, AKT3, and STAT6; as well as upregulation of CDKN1A and E2F7 after miR-221/222 inhibitor plus melphalan treatment compared with either untreated or single-agent treated U266/LR7 cells. Altogether, these findings define a transcriptome profile that underlies the response to the combination treatment in multiple myeloma cells, suggesting further investigation of miR-221/222 involvement in resistance mechanisms.

**PUMA/BBC3 is a target of miR-221/222 in multiple myeloma cells**

We validated GEP data on XRCC4, CDKN1A, PRKDC, SLC7A8/LAT2, SLC7A5/LAT1, ABCC1/MRP1, and PUMA/BBC3 by qRT-PCR (Supplementary Figs. SSA and SSB). In silico search for target prediction using miDIP software indicates Bel-2-binding component 3, PUMA/BBC3, as a strongly predicted target of miR-221/222. On the basis of target prediction, we cotransfected U266/LR7 cells with synthetic miR-221/222 mimics together with firefly luciferase constructs, in which either the WT or mutant 3′-UTR of PUMA/BBC3 mRNA are cloned downstream. A marked decrease in luciferase activity (42% for miR-221 and 38% for miR-222) indicated direct interactions between the miRNAs and PUMA/BBC3 3′-UTR; moreover, target gene repression was rescued by deletions in the mutant clone (Fig. 4A). These findings provide validation of direct miR-221/222 targeting of 3′-UTR of PUMA/BBC3 mRNA in multiple myeloma cells.

**miR-221/222 inhibitors upregulate PUMA/BBC3 protein levels triggering melphalan sensitivity in drug-resistant multiple myeloma cells**

We next analyzed PUMA/BBC3 expression both at the mRNA and protein levels in U266/LR7 cells transfected with miR-221/222 inhibitors, and then exposed to melphalan. By Western blotting, a strong upregulation of PUMA/BBC3 protein was found in cells treated with miR-221/222 inhibitors plus melphalan; conversely, exposure to melphalan alone led to upregulation of PUMA/BBC3 at mRNA level, but not at protein level (Fig. 4B). Moreover, enforced expression of synthetic miR-221/222 mimics in melphalan-treated U266/S cells abrogates PUMA/BBC3 protein translation induced by melphalan alone (Fig. 4C). To evaluate the role of PUMA overexpression in enhancing melphalan activity, we exposed the U266/LR7 cells transfected with miR-221/222 inhibitors together with siPUMA or sICNT to melphalan. Importantly, in resistant cell lines silenced for PUMA/BBC3 (Fig. 4D, right), we found a significant antagonism (30%) of the sensitizing activity of miR-221/222 inhibitors compared to cotransfection with sICNT (Fig. 4D, left). As a further confirmation, PUMA/BBC3 silencing led to a 20% reduction in melphalan activity of in-drug-sensitive U266/S cells (Fig. 4E, right). Based on these findings, we conclude that the sensitizing activity of miR-221/222 inhibitors to melphalan in multiple myeloma cells is, at least in part, dependent on PUMA/BBC3 protein upregulation.

**miR-221/222 inhibition modulates expression of drug influx–efflux transporters in multiple myeloma cells**

Since GEP analysis showed modulation of drug transporter mRNA transcripts, we next studied the effects of miR-221/222 inhibitors plus melphalan on their expression. L-type amino acid transporter SLC7A5/LAT1 and ABC transporter ABCC1/MRP1 were evaluated at the protein level at miR-221/222 inhibitor or scramble transfected U266/LR7 cells. As shown in Fig. 5A, miR-221/222 inhibition plus melphalan resulted in a significant upregulation of SLC7A5/LAT1 protein, as well as a marked downregulation of ABCC1/MRP1 protein (Fig. 5A). Moreover, based on the known ability of ABCC1/MRP1 pump to exclude drugs as well as fluorescent dyes such as Hoechst 33342 (41), we next evaluated by Hoechst dye exclusion assay whether miR-221/222 inhibitor treatment may affect dye cellular uptake. As expected, the cell fraction in U266/LR7 cells that exhibits low level of Hoechst fluorescence intensity, identified as side population (SP), decreased by 65% after treatment with miR-221/222 inhibitors, and by 74% after treatment with ABC transporters inhibitor verapamil (Fig. 5B and C). Taken together, these findings identified a role of miR-221/222 in regulation of drug transporter expression.

**LNA-i-miR-221 inhibitors overcome melphalan-induced drug resistance in multiple myeloma cells in vivo**

Finally, we evaluated the antitumor activity of the combination in NOD/SCID mice bearing human melphalan-resistant multiple myeloma xenografts in two independent in vivo studies. For each study, animals were randomized to receive four different treatments. As shown in Fig. 6, i.p. treatment with LNA-i-miR-221 (25 mg/kg) on days 1, 4, 8, 15, and 22 plus melphalan (0.75 mg/kg) twice weekly for 3 weeks overcame drug resistance, as evidenced by a significant tumor growth inhibition compared with control groups (P < 0.05; Fig. 6A). In a subsequent in vivo study, we optimized the treatment schedule to improve the sensitizing activity of LNA-i-miR-221 and to adapt the LNA-i-miR-221 administration to a common melphalan schedule used in multiple myeloma patients. Specifically, the treatment with LNA-i-miR-221 plus melphalan was given consecutively for 4 days every 10 days. This approach strengthened the antitumor activity of the combination (P < 0.001; Fig. 6B). Moreover, no changes in body weight or mice behaviors were observed; H&E staining on retrieved organs from treated animals did not show any organ toxicity (data not shown). Additionally, and consistent with our in vitro findings, analysis of retrieved tumors confirmed upregulation of PUMA/BBC3 protein (Fig. 6C, bottom) and inhibition of ABCC1/MRP1 protein (Fig. 6C, top) in mice treated with LNA-i-miR-221 plus melphalan. IHC analysis on excised tumors demonstrated extended necrosis following combination treatment, with reduction in Ki-67 proliferation index (Fig. 6D). Altogether, these in vivo findings further indicate the translational relevance of LNA-i-miR-221 as sensitizing agents in melphalan-resistant multiple myeloma.

**Discussion**

Targeting the miRNA network is arising as a new promising strategy to overcome drug resistance of human cancer (42). We...
LNA-i-miR-221 Inhibitor Overcomes Melphalan Resistance

Previously showed that inhibition of oncogenic miR-221/222 in multiple myeloma cell lines induces significant anti-multiple myeloma activity via targeting key molecules involved in cell proliferation and survival (34), and that LNA-inhibitors of miR-221 are suitable for safe and effective systemic delivery in mice (35). We here report that in vitro inhibition of miR-221/222 restores melphalan sensitivity, inducing antiproliferative and apoptotic cell death in drug-refractory multiple myeloma cells. More importantly, this antitumor activity has been also shown in vivo following systemic treatment with LNA-i-miR-221 plus melphalan in SCID/NOD mice bearing melphalan-resistant multiple myeloma xenografts without any evidence of toxicity or side effects in treated mice. Our results further support the role of miR-221/222 as crucial mediator of tumor cell resistance to alkylating agents such as cisplatin and temozolamide (28, 31), and we here provide proof-of-principle that LNA-i-miR-221 is a potent sensitizing-agent in melphalan-refractory multiple myeloma.

To investigate the mechanistic role of miR-221/222 in melphalan-resistant multiple myeloma, we first assessed the correlation between miR-221/222 expression and the antiproliferative activity of melphalan. Decreased miR-221/222

Figure 4.
Inhibition of miR-221/222 enhances anti-multiple myeloma activity of melphalan by modulation of PUMA/BBC3 expression. A, dual-luciferase assay of U266/LR7 cells cotransfected either with NC or with synthetic miR-221/222 mimics, together with firefly luciferase constructs containing the wild-type or mutant 3’ UTR of PUMA/BBC3 mRNA. The firefly luciferase activity was normalized to Renilla luciferase activity. Data are represented as relative luciferase activity of either miR-221 or miR-222 mimics electroporated cells as compared with control. Values represent the mean ± SD of three independent experiments. *, P < 0.05. B, qRT-PCR (bottom) and immunoblot (top) of PUMA/BBC3 in resistant U266/LR7 cells after transfection with NC or miR-221/222 inhibitors, and treatment with 100 μmol/L of melphalan. qRT-PCR results are shown after normalization with GAPDH and ∆∆Ct calculation, and represent an average ± SD of three independent experiments. Protein loading control for immunoblot was performed using GAPDH. (M = melphalan). C, qRT-PCR (bottom) and immunoblot (top) of PUMA/BBC3 in sensitive U266/S cells after transfection with NC or miR-221/222 mimics, and treatment with 60 μmol/L of melphalan. qRT-PCR results are shown after normalization with GAPDH and ∆∆Ct calculation, and represent an average ± SD of three independent experiments. Protein loading control for immunoblot was performed using γ-tubulin (γ-TUB). (M = melphalan). D, CCK-8 cell proliferation assay on resistant U266/LR7 cells (left panel) treated with either NC or miR-221/222 inhibitors cotransfected with siCNT or siBBC3/PUMA and subsequently exposed to 100 μmol/L of melphalan. Effective knockdown was confirmed by immunoblot of PUMA/BBC3 protein and γ-tubulin normalization (right). Percentages of growth inhibition are plotted compared with control. Effective knockdown was confirmed by immunoblot of PUMA/BBC3 protein and γ-tubulin normalization (right). Cell viability is shown as percentage of control. Values represent the mean ± SD of three independent experiments. *, P < 0.05.
expression after melphalan treatment in sensitive cells along with increased expression in resistant cells suggested a correlation of miR-221/222 with melphalan-resistance. This observation was also confirmed by the basal higher expression of both miRNAs premelphalan treatment in U266/LR7 cells as compared with the parental sensitive counterpart U266/S. Given that miR-221/222 expression has been shown to be activated by transcription factor c-Jun likely as DNA-damage response following radiation therapy (43), it is tempting to hypothesize that a similar mechanism may occur in multiple myeloma cells after melphalan exposure. Follow-up studies will be carried out to clarify this point.

Indeed, inhibition of miR-221/222 markedly overcame melphalan resistance in U266/LR7, RPMI-8226, and AMO1 Abzb, evidenced by a strong synergism of these two agents; conversely, overexpression of these miRNAs antagonizes drug-activity in melphalan-sensitive U266/S cells. Moreover, the synergistic effect of the combination affected the survival of primary cells of multiple myeloma patients without any cytotoxicity in PBMCs from healthy donors, suggesting a low-toxicity profile of our approach. In our in vitro experimental model, miR-221/222 inhibitors enhance melphalan-induced apoptosis, as confirmed by activation of caspase cascade. Moreover, we found that upregulation of miR-221/222 induced by adhesion of multiple myeloma cells to BMSCs does not impair the effectiveness of this combination, indicating that this treatment can overcome the role of the multiple myeloma microenvironment in promoting resistance to DNA-damaging agents (44).

Interestingly, functional analysis of genes and pathways significantly modulated in resistant U266/LR7 cells demonstrated a remarkable similarity in gene expression signatures of melphalan-resistant U266/LR7 multiple myeloma cells treated with miR-221/222 inhibitors plus melphalan compared with the sensitive parental U266/S cells treated with melphalan. Notably, IPA analysis demonstrated significant perturbation of pathways of central relevance in cellular response to alkylating agents, frequently correlated with drug-resistance such as "NRF2-mediated oxidative stress response" (45), "cell cycle: G2/M DNA damage checkpoint regulation," "DNA DSB repair by nonhomologous end joining" (46), as well as those pathways associated with tumor progression. Based on GEP data, we focused on proapoptotic PUMA/BBC3, since its downregulation has been associated with drug resistance (47, 48) and miR-221/222-sensitizing activity to temozolomide (31). After luciferase reporter assay validation of PUMA/BBC3 mRNA as a specific miR-221/222 target, we demonstrated that miR-221/222 inhibitors induce mRNA and protein PUMA expression. Conversely, PUMA/BBC3 silencing by siRNA transfection in melphalan-resistant U266/LR77 cells antagonized the melphalan-sensitizing activity of miR-221/222 inhibitors. Of note, PUMA/BBC3 silencing also led to a significant reduction in melphalan activity in drug-sensitive U266/S cells. Our results therefore highlight the role of PUMA as key player in miR-221/222-mediated induction of melphalan resistance.

Several reports have emphasized the role of ATP-dependent ABC transporters which rapidly efflux several drugs as crucial...
determinants of multidrug resistance in cancer cells (49). Of note, aberrant expression of influx and efflux transporters has been correlated with altered sensitivity to melphalan (50). Interestingly, our GEP data suggested the activity of miR-221/222 targeting ATP-dependent efflux MRP1/ABCC1 as well as influx SLC7A5/LAT1 transporters, and we here confirmed that miR-221/222 inhibitors upregulate LAT1 and downregulate ABCC1 at the protein level. Furthermore, a subset of cancer stem-like cells, identified as "SP," expresses these transporters, which correlates with chemoresistance and a high tumorigenic potential (41). In this light, we performed a functional analysis to characterize cells that contribute to the SP phenotype, and we show a marked decrease of SP in cells treated with miR-221/222 inhibitors. In silico search for target prediction using mirDIP software did not indicate direct targeting of mRNAs, and further studies are necessary to solve the mechanism of miR-221/222 activity on LAT1 and ABCC1 transporters. Nonetheless, our results are of translational relevance since they provide proof of concept that miR-221/222 inhibition can antagonize resistance to melphalan through modulation of influx–efflux transporters. Finally, we validated our in vitro findings in murine xenograft models with human melphalan-resistant multiple myeloma cells in two independent experiments: a stronger antitumor activity has been achieved with consecutive 4 days of treatment with LNA-i-miR-221 plus melphalan. This effect was associated with upregulation of PUMA/BBC3 and downregulation of ABCC1 proteins in tumors harvested from treated animals. In conclusion, the validation of LNA-i-miR-221 as a melphalan-sensitizing agent both confirms the role of miR-221/222 in mediating drug resistance and provides the framework for

Figure 6. LNA-i-miR-221 Enhances Anti-multiple Myeloma Activity of Melphalan Overcoming Drug Resistance. For the in vivo study, mice xenografted with U266/LR7 cells were randomized in four groups of treatment. A, treatments were: (i) i.p. LNA-i-miR-221 (25 mg/kg) at day 1, 4, 8, 15, and 22; (ii) scrambled control with the same schedule; (iii) i.p. melphalan (0.75 mg/kg) on days 2, 5, 9, 12, 16, 19; (iv) melphalan plus LNA-i-miR-221 with the above described schedules. B, mice were treated with: (i) i.p. LNA-i-miR-221 (25 mg/kg) or (ii) scramble control or (iii) i.p. melphalan (0.75 mg/kg) or (iv) melphalan plus LNA-i-miR-221 for consecutive 4 days every 10 days (1-4, 15-18). Arrows indicate the days of treatment. Tumors were measured with an electronic caliper every 2 days, and data are represented as averaged tumor volume ± SD of each group. P values were obtained using two-tailed t test and calculated by comparing LNA-i-miR-221 plus melphalan group compared with each of the others. The pictures inserted show the in vivo detection of the tumor volume in a representative mouse of each group using IVIS LUMINA II Imaging System. ∗, P < 0.05; ∗∗, P < 0.001. C, Western blot analysis of PUMA/BBC3 and ABCC1 levels in lysates from a representative retrieved xenograft from each treatment group. ABCC1/MRP1 was immunoprecipitated from cell lysates (3 mg) following incubation with 30 μL of protein A/G-conjugated agarose beads at 4°C overnight. Immunoprecipitated ABCC1/MRP1 was detected by Western blot. Gamma-Tubulin was used as protein loading control. D, H&E (<400) and Ki67 (<400) IHC analysis of retrieved xenografted tumors after different treatments. Representative images from each group are shown.
combination clinical trials to sensitize or overcome resistance to melphalan in multiple myeloma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Disclaimer**

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**References**

A 13 mer LNA-i-miR-221 Inhibitor Restores Drug Sensitivity in Melphalan-Refractory Multiple Myeloma Cells

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