Human Multiple Myeloma Cells Are Sensitized to Topoisomerase II Inhibitors by CRM1 Inhibition

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Abstract

Topoisomerase IIα (topo IIα) is exported from the nucleus of human myeloma cells by a CRM1-dependent mechanism at cellular densities similar to those found in patient bone marrow. When topo IIα is trafficked to the cytoplasm, it is not in contact with the DNA; thus, topo IIα inhibitors are unable to induce DNA-cleavable complexes and cell death. Using a CRM1 inhibitor or a CRM1-specific small interfering RNA (siRNA), we were able to block nuclear export of topo IIα as shown by immunofluorescence microscopy. Human myeloma cell lines and patient myeloma cells isolated from bone marrow were treated with a CRM1 inhibitor or CRM1-specific siRNA and exposed to doxorubicin or etoposide at high cell densities. CRM1-treated cell lines or myeloma patient cells were 4-fold more sensitive to topo IIα poisons as determined by an activated caspase assay. Normal cells were not significantly affected by CRM1-topo II inhibitor combination treatment. Cell death was correlated with increased DNA double-strand breaks as shown by the comet assay. Band depletion assays of CRM1 inhibitor–exposed myeloma cells showed increased topo IIα covalently bound to DNA. Topo IIα knockdown by a topo IIα–specific siRNA abrogated the CRM1-topo II therapy synergistic effect. These results suggest that blocking topo IIα nuclear export sensitizes myeloma cells to topo IIα inhibitors. This method of sensitizing myeloma cells suggests a new therapeutic approach to multiple myeloma.

Introduction

Drug resistance is a major obstacle in the treatment of multiple myeloma, including resistance to topoisomerase II (topo II) inhibitors. Topo II poisons that are used in the treatment of multiple myeloma include doxorubicin and etoposide (VP-16). Several mechanisms of resistance to topo IIα inhibitors have been described (reviewed previously in refs. 1–5). Cell adhesion–mediated drug resistance and stromal cell adherence are important parameters in the local bone marrow environment in patients with multiple myeloma and appear to be major determinants of drug resistance (6, 7). Our laboratory has shown previously that human multiple myeloma cell density is a determinant of sensitivity to topo IIα inhibitors (8–10). At increased cell densities, a considerable fraction of nuclear topo IIα is exported to the cytoplasm (>90%), resulting in reduced sensitivity to VP-16 and doxorubicin. This appears to occur both in human myeloma cell lines and in CD138+ cells isolated from patients with multiple myeloma (9). We have reported that the nuclear export of topo IIα may contribute to drug resistance (9), and our data suggest that resistance is not due to differences in drug uptake, cell cycle, or cellular topo IIα protein levels (8–10). In a previous report, our group defined the nuclear export signals for topo IIα at amino acids 1,017 to 1,028 and 1,054 to 1,066 (10). Export by both signals was blocked by treatment of the cells with leptomycin B, indicating that a CRM1-dependent pathway mediates export (10). In the present study, we show that inhibition of CRM1-mediated export of topo IIα may render myeloma cells both in vitro and ex vivo more sensitive to topo IIα–targeted chemotherapy.

Use of CRM1 inhibition in cancer therapy has met with limited success. The first CRM1 inhibitor, leptomycin B, was found to efficiently inhibit nuclear export (11). However, leptomycin was found to have acute relative toxicities both in a human phase I trial (12) and in vitro (13). Leptomycin B in vitro studies found acute toxicity at concentrations <5 nmol/L for 1 h (13). Therefore, in this study, we used the CRM1 inhibitor ratjadone C (14–18). Ratjadone C has been found to inhibit nuclear export without producing apoptosis or necrosis at concentrations up to 300 nmol/L for 48 h in an in vitro assay (18). However, ratjadone C prevents nuclear export of topo IIα; in this study, we show that ratjadone C also acutely sensitizes myeloma cells to the topo IIα inhibitors doxorubicin and VP-16. Additional low-toxicity CRM1 inhibitors are also being investigated by other laboratories and may become available for preclinical studies (13).

Materials and Methods

Cell lines. Human myeloma cell lines NCI-H929 (H929) and RPMI-8226 (8226) were newly obtained from the American Type Culture Collection. U266B1 (U266) human myeloma cell lines were provided by Dr. Lori Hazlehurst (H. Lee Moffitt Cancer Center). All cell lines were grown in RPMI 1640 containing 100 units/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 10% fetal bovine serum (Hyclone) at 37°C and 5% CO2. H929 cell medium required the addition of 0.025% β-mercaptoethanol (Sigma).

Cell density and drug treatment. The model used to assay density-dependent protein trafficking involved incubating cells at high- and low-density culture conditions. Our group has shown previously that cells grown at different densities exhibit specific characteristics such as drug resistance and nuclear-cytoplasmic trafficking of topo IIα (8–10). Human myeloma cell lines (8226, H929, and U266) grown at 2 × 106 cells/mL were defined as low density (log phase), and cells grown at 2 × 106 cells/mL were defined as high density (plateau phase). Cell lines were placed at log- and plateau-density conditions and incubated with and without the highly specific CRM1 inhibitor ratjadone C (refs. 14–18; Department of Chemical Biology, HZI/Helmholtz Centre for Infection Research) or were transfected with
CRM1 200 nmol/L small interfering RNA (siRNA; Dharmaco). Ratjadones are naturally occurring antibiotics isolated from myxobacteria (16). Ratjadone C is a potent inhibitor of CRM1 and prevents nuclear export by alkylating the active site Cys298 amino acid residue of CRM1 (17). Ratjadone C has been shown to have antitumor properties and has reduced toxicity in vitro compared to leptomycin B (14, 16, 18). To determine the concentration of ratjadone C to use, we titrated ratjadone C to find the lowest concentration that would inhibit nuclear export of topo IIα in myeloma cell lines H929 and 8226. The concentration arrived at was 5 nmol/L all ratjadone C experiments used this concentration. Cells were treated with ratjadone C for 16 h followed by doxorubicin (2 μmol/L Sigma) for 4 h or VP-16 (10 nmol/L; Sigma) for 8 h and assayed for apoptosis by either anti-caspase-3 or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining (BD Pharmingen).

**Figure 1.** Intracellular trafficking of topo IIα in log- and plateau-density myeloma cells. A, H929, U266, and 8226 human myeloma cells grown at plateau (Plat) phase (high density) export topo IIα, whereas cells grown at log (Log) phase (low density) maintain topo IIα in the nucleus. Cells were grown for 16 h at log or plateau densities and treated with 2 μmol/L doxorubicin for 4 h (n = 2). Apoptosis was determined by caspase-3 staining with the use of flow cytometry (10,000 cells). Cells that maintained nuclear topo IIα were more sensitive to topo II-targeted chemotherapy. B, cells grown at log- or plateau-phase conditions or cells treated with ratjadone C (RDC; 100 cells per experiment) were stained for topo IIα by fluorescence microscopy (n = 2). Myeloma cells grown at log-phase conditions had the majority (>90%) of the topo IIα in the nucleus, whereas plateau-phase cells exported topo IIα into the cytoplasm. The CRM1 inhibitor ratjadone C (5 nmol/L) was found to block export of topo IIα in cells grown in plateau-phase conditions.

**CRM1 inhibitor sensitizes patient myeloma cells to topo II drugs.** Human bone marrow aspirates from multiple myeloma patients were collected using a protocol approved by the University of South Florida Institutional Review Board. Patient samples were assayed for percentage of plasma cells by toluidine blue staining and microscopy. Cells used for assay consisted of between 75% and 90% plasma cells isolated by Ficoll gradient centrifugation. Cells (4 × 10⁶/mL) were treated with ratjadone C (5 nmol/L) for 16 h followed by doxorubicin (2 μmol/L) for 4 h and assayed for apoptosis by anti-caspase-3 (BD Pharmingen).

**siRNA knockdown and Western blot.** All electroporation transfections were done in a freshly made transfection buffer containing 120 mmol/L potassium chloride (Sigma), 0.15 nmol/L calcium chloride, 10 mmol/L potassium phosphate (pH 7.6), 25 mmol/L HEPES, 2 mmol/L EGTA (pH 7.6), 5 mmol/L magnesium chloride, 2 mmol/L ATP (pH 7.6), 5 mmol/L glutathione, 1.25% DMSO, and 50 mmol/L trehalose (Sigma). Each transfection used 3 × 10⁶ myeloma cells. Cells were washed two times in PBS and placed in a 200 μL volume of transfection buffer. CRM1-specific siRNA (Dharmacon), scrambled control siRNA (Dharmacon), or topo IIα-specific siRNA (Ambion) was added (200 nmol/L); the sample was then placed in a 2 mm electroporation cuvette and transfected at 140 V and 975 μF in a Bio-Rad GenePulser Xcell electroporation unit (Bio-Rad). Transfected cells were incubated in the cuvette for 15 min at 37°C in a 5% CO₂ incubator and transferred to a sterile T25 tissue culture flask, and 10 mL fresh medium was added. After 48 h, the transfected cells were harvested by centrifugation at 500 g for 5 min, washed with cold PBS, and lysed by sonication (40% duty cycle, 7 bursts) in SDS buffer [2% SDS, 10% glycerol, 60 mmol/L Tris (pH 6.8); Protein from 2 × 10⁵ cells per lane was separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Amersham) overnight (30 V at 4°C) with the use of a Bio-Rad Mini-Transblot apparatus. Membranes were blocked for 1 h at ambient temperature in a blocking buffer containing 0.1 mol/L Tris-HCl, 0.9% NaCl, and 0.5% Tween 20 (TBST) and 5% nonfat dry milk. CRM1 was identified by incubation in a 1:1,000 dilution of H-300 antibody (Santa Cruz Biotechnology) in blocking buffer overnight at 4°C. Membranes were washed three times for 10 min in TBST and incubated for 1 h with goat anti-rabbit polyclonal IgG antibody linked to a horseradish peroxidase antibody (Sigma) in blocking buffer at a 1:2,000 dilution. Antibody binding was visualized by enhanced chemiluminescence (Amersham) on autoradiography film (Kodak). Transfected cells were treated with doxorubicin (2 μmol/L) for 4 h and assayed for apoptosis by Annexin V-FITC staining (BD Pharmingen).

**Immunofluorescent microscopy.** Multiple myeloma cells (1 × 10⁶) were plated on double coverslides (Shandon) by cyto-centrifugation at 500 rpm for 3 min and fixed with 1% paraformaldehyde (Fisher Scientific) on ice for 30 min. Permeabilization of cells was done with 0.5% Triton X-100 (Sigma) in PBS at room temperature for 60 min. Cells were stained with a polyclonal antibody against topo IIα, which was produced in our laboratory (PAB454; ref. 19). The topo IIα antibody was diluted 1:100 in a buffer...
containing 1% bovine serum albumin (Sigma) and 0.1% Igepal CA-630 (Sigma) in PBS and incubated for 1 h at room temperature. After three washes with PBS, slides were incubated with a secondary anti-rabbit Alexa Fluor 594 (Invitrogen) in addition to a cytoskeletal protein stain, phalloidin-Alexa Fluor 488 conjugate (Invitrogen). Each was diluted 1:1,000 in 1% bovine serum albumin and 0.1% Igepal CA-630 in PBS and incubated for 40 min at room temperature. Slides were washed four times in PBS and once in distilled water, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Immunofluorescence was observed with the Zeiss Axio Imager Z1 microscope (Carl Zeiss Microimaging) with an AxioCam MRm camera (Carl Zeiss Microimaging). Two experiments were done with 50 cells assayed per experiment. Cells were chosen randomly and scored as nuclear or cytoplasmic when ≥90% of the fluorescence was in the respective cellular compartment.

**Band depletion assay.** Band depletion assays were done as described by Xiao and colleagues (20). Briefly, 5 × 10^3 cells were lysed in 50 µL alkaline lysis solution for 30 min on ice (200 mmol/L NaOH, 2 mmol/L EDTA), and the lystate was neutralized by the addition of 4 µL of both 1 mol/L HCl and 1.2 mol/L Tris (pH 8.0). The lysate was then mixed with 30 µL of 3× SDS sample buffer [150 mmol/L Tris-HCl (pH 6.8), 6 mmol/L EDTA, 45% sucrose, 9% SDS, and 10% [β-mercaptoethanol] and separated on 8% SDS-PAGE gels.

**Comet assay.** Log-density H929 myeloma cells were plated at a concentration of 2 × 10^5/mL and plateau-density cells were plated at 2 × 10^6/mL. All cells were grown in 24-well plates (Falcon) with 1 mL sample/well. Drug treatment groups were vehicle only (1 µL/L DMSO), 10 µmol/L VP-16, 5 nmol/L ratjadone C, or a combination of 10 µmol/L VP-16 and 5 nmol/L ratjadone C. Cells that were treated with ratjadone C were first plated at log or plateau density and incubated for 16 h with ratjadone C or vehicle, after which VP-16 was added for 1 h. After 1 h of VP-16 exposure, the comet assay was done as described by Kent and colleagues (21) and modified by Chen and colleagues (22). To ensure random sampling, 50 images were captured per slide on a Leica fluorescent microscope and quantified with ImageQuant software (Molecular Dynamics). The average comet moment value obtained from vehicle control samples was subtracted from the average comet moment of each drug treatment sample. The data shown are mean ± SD of two separate experiments.

**Additional drug combinations in H929 human myeloma cell lines.** H929 human myeloma cell lines were placed at high density (4 × 10^6) with and without 5 nmol/L ratjadone C and incubated for 16 h. Cells were then incubated for 4 h with one of the following: 2 µmol/L doxorubicin, 10 µmol/L VP-16, 10 µmol/L melphalan, or 10 µmol/L Velcade. Cells were assayed for apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining.

**Results**

**Log- and plateau-density myeloma cells.** The myeloma cell lines 8226, H929, and U266 were grown at log-density (2 × 10^5 cells/mL) and plateau-density (2 × 10^6 cells/mL) growth conditions for 16 h. Cells were then treated with the topo II inhibitor doxorubicin (2 µmol/L) for 4 h and assayed for apoptosis by activated caspase-3 expression. We found that cells grown at plateau densities and treated with 2 µmol/L doxorubicin had extremely low levels of apoptosis compared with results shown for log-phase cells (Fig. 1A). These data confirmed previous results that showed that cells grown at different densities exhibit specific characteristics, such as drug resistance and nuclear-cyttoplasmic trafficking of topo IIα (8–10). The topo IIα isozyme, topo IIβ, is not exported from the nucleus in human myeloma cells (8).

**Intracellular trafficking of topo IIα.** Cells grown at log and plateau densities and ratjadone C–treated cells (100 cells per experiment) were scored as “nuclear” or “cytoplasmic” if ≥90% of topo IIα was in that compartment as determined by fluorescence microscopy (Fig. 1B). For myeloma cells grown at log-phase concentrations, 73% to 87% of cells had ≥90% of the topo IIα in the nucleus; for cells grown at plateau-phase concentrations, 85% to 93% of cells had ≥90% of the topo IIα in the cytoplasm. CRM1 inhibition by ratjadone C was found to block export of topo IIα in cells grown in plateau-phase conditions (Fig. 1B).

**Topo IIα trafficking and CRM1 inhibition.** H929 human myeloma cells were grown at log and plateau densities and stained for cytoskeletal protein (phalloidin; green), topo IIα (red), and DNA (DAPI; blue). Results indicate that topo IIα was present in the nucleus of log-density cells and was exported from the nucleus in plateau-density cells (Fig. 2). Nuclear export was blocked in plateau cells by the CRM1 inhibitor ratjadone C and by transfection with a CRM1-specific siRNA. Under the conditions of this experiment, CRM1 siRNA knockdown was 69%. For ratjadone C–treated plateau-density cells (Fig. 1B), ~70% of the cells had ≥90% topo IIα in the nucleus for each myeloma cell line.

**CRM1 inhibitor and topo IIα inhibitor synergy.** The myeloma cell lines 8226, H929, and U266 were grown for 16 h at plateau densities in the presence of ratjadone C (5 nmol/L). The cells were
CRMA inhibitor sensitizes myeloma cells to topo II drugs. Human bone marrow aspirates were obtained from multiple myeloma patients and purified by Ficoll-Paque gradient. Bone marrow samples with >75% plasma cells, as determined by toluidine blue staining and microscopy, were used for each assay (n = 7). Cells were placed at plateau concentration (4 × 10^6/mL), treated with ratjadone C (5 nmol/L) for 16 h, followed by doxorubicin (2 μmol/L) for 4 h, and assayed for cleaved caspase-3 to determine apoptosis. Cells treated with ratjadone C were significantly (P = 0.0003) more sensitive to doxorubicin (3-fold) than doxorubicin alone (Fig. 3B). Normal cells, including flow 2,000 cells and peripheral blood mononuclear cells (n = 5), were not sensitized by the CRMA inhibitor (P = 0.22). Figure 3B shows peripheral blood mononuclear cells only.

CRMA inhibitor and topo IIα poisons. The human myeloma cell lines 8226, H929, and U266 were incubated at high density (2 × 10^6 cells/mL) for 16 h in the presence of the CRMA inhibitor ratjadone C (5 nmol/L). Cell cultures were then exposed to the topo II–targeted agents VP-16 (10 μmol/L) for 8 h or doxorubicin (2 μmol/L) for 4 h and assayed for apoptosis with a caspase-3 assay (BD Pharmingen). In addition, to show whether the ratjadone C/doxorubicin and the ratjadone C/VP-16 synergistic activities were because of topo IIα nuclear localization, cells were transfected with a siRNA to knockdown topo IIα expression (Fig. 3C). Topo IIα knockdown was >90% when assayed by Western blot (Fig. 3C, inset). In myeloma cell lines, with both topo II inhibitors (doxorubicin and VP-16), we found that knockdown of topo IIα protein expression reversed the synergistic effect and reduced apoptosis to untreated levels (4-12% apoptosis; Fig. 3C).

CRMA siRNA sensitizes myeloma cells to topo IIα poisons. In addition to CRMA pharmacologic modification by a chemical agent, we used a CRMA-specific siRNA to determine whether we could reproduce the observed synergistic activity in another model.

Figure 3. CRMA inhibitor sensitizes myeloma cells to doxorubicin. A, human myeloma cell lines 8226, H929, and U266 (2 × 10^6 cells/mL) were incubated with the CRMA inhibitor ratjadone C (RDC; 5 nmol/L) for 16 h. Cells were then treated with doxorubicin (Dox; 2 μmol/L) for 4 h and assayed for caspase-3 staining by flow cytometry. Cell lines were assayed in triplicate, and data from each drug combination were pooled. Ratjadone C versus control samples are statistically different (P = 0.006) in A (control = 1.50% and ratjadone C = 3.84%). Ratjadone C was found to significantly (P = 0.0005) sensitize cells to doxorubicin. B, CRMA inhibitor sensitizes ex vivo patient myeloma cells to doxorubicin. Bone marrow aspirates (n = 7) obtained from multiple myeloma patients were treated with ratjadone C (5 nmol/L) for 16 h followed by doxorubicin (2 μmol/L) for 4 h and assayed for caspase-3 staining by flow cytometry. Cell lines were assayed in triplicate, and data from each drug combination were pooled. Ratjadone C versus control samples are statistically different (P = 0.03) from untreated cells (control = 4.97% and ratjadone C = 14.31%). Cells (4 × 10^6/mL) treated with both ratjadone C and doxorubicin were significantly (P = 0.0003) more sensitive to doxorubicin (3-fold) than doxorubicin alone, and the drug combination was >90% when assayed by Western blot (Fig. 3C, inset). In myeloma cell lines, with both topo II inhibitors (doxorubicin and VP-16), we found that knockdown of topo IIα protein expression reversed the synergistic effect and reduced apoptosis to untreated levels (4-12% apoptosis; Fig. 3C).

Figure 4. CRMA knockdown using siRNA makes myeloma cells more sensitive to the topo II poison doxorubicin. H929 cells were transfected by electroporation with a CRMA-specific siRNA or scrambled siRNA (Scram) control. After transfection, the cells were incubated at log-phase density for 24 h to allow CRMA siRNA-mediated knockdown and concentrated at the plateau-phase condition for an additional 16 h. CRMA knockdown cells grown at high density for 16 h were then treated with the topo II inhibitor doxorubicin (2 μmol/L) for 4 h and assayed for apoptosis with Annexin V/propidium iodide (PI) staining with the use of flow cytometry. CRMA knockdown rendered plateau-density cells more sensitive to topo II α inhibitors. Inset, Western blot data for siRNA transfection. Percent knockdown, compared with control siRNA, ranged between 60% and 65%.
H929 cells were transfected by electroporation with a CRM1-specific siRNA (Fig. 4). After transfection, the cells were incubated at log-phase densities for 24 h to allow CRM1 siRNA-mediated knockdown and then concentrated at plateau-phase conditions for an additional 16 h. CRM1 knockdown cells grown at high density for 16 h were then treated with the topo II inhibitor doxorubicin (2 μmol/L) for 4 h and assayed for apoptosis by Annexin V staining using flow cytometry (Fig. 4). CRM1 knockdown was found to increase the effectiveness of doxorubicin. Myeloma cell line 8226 showed similar results (data not shown). To show efficient siRNA knockdown, SDS lysates of equal cell numbers were assayed for CRM1 by Western blot (Fig. 4, inset).

**Increase in cleavable complex formation by CRM1 inhibition.** VP-16 stabilizes DNA-topo II adducts, resulting in double-stranded DNA breaks. We speculated that, when topo II is kept in the nucleus by ratjadone C, a greater number of DNA-topo II complexes would be observed. To assay this potential effect, several band depletion assays were done \( (n = 3) \). Band depletion assays can assess the amount of topo II-DNA covalent complexes formed in intact cells. Large covalent complexes will have decreased migration into a SDS-PAGE gel; therefore, the amount of topo II, as measured by Western blot, will be depleted. VP-16 alone did not produce significant band depletion; however, when used together with ratjadone C, we saw a large depletion at both 25 and 50 μmol/L VP-16 concentrations (Fig. 5A). These data indicate that blocking nuclear export of topo II will increase the effectiveness of VP-16 and induce apoptosis by increased cleavable complexes.

**Comet assay.** Plateau-density H929 cells were treated with 5 nmol/L ratjadone C for 16 h and then with 10 μmol/L VP-16 for 60 min. DNA fragmentation was measured by the neutral comet assay. The CRM1 inhibitor ratjadone C increased the DNA cleavage induced by the topo II inhibitor VP-16 (Fig. 5B). Increased DNA fragmentation led to increased apoptosis in cells treated with both VP-16 and ratjadone C.

**Ratjadone C does not sensitize H929 human myeloma cell lines to other myeloma drugs.** H929 myeloma cells were exposed to ratjadone C and various additional drug combinations (Fig. 6), including the alkylating agent melphalan and proteosome inhibitor Velcade. We found that CRM1 inhibition will sensitize myeloma cells to the topo II inhibitors doxorubicin \( (P = 0.00005) \) and VP-16 \( (P = 0.02) \), but it did not significantly sensitize myeloma cells treated with melphalan \( (P = 0.35) \) or Velcade \( (P = 0.30) \).

**Discussion**

The intracellular location of a protein may be at least as important as its expression. Diseases as dissimilar as cystic fibrosis (23), schizophrenia (24), nephrogenic diabetes insipidus (25), and many types of cancers, as reviewed previously (26, 27), may be...
caused by intracellular mislocalization of individual proteins. Specific examples of molecules that must be in the nucleus to prevent cancer initiation, progression, or chemotherapeutic response include p53 (reviewed previously in ref. 28), galectin-3 (29), FOXO (30), IN1/HsSNPF5 (31), p23 Kirp (32), p21 Cip1 (33), and topo IIa (8–10). Mislocalization of a protein can render it ineffective as a tumor suppressor or as a target for chemotherapy. However, it is possible that blocking nuclear export of any or all of these proteins may induce tumor suppression or apoptosis or, in the case of topo IIa, may reverse drug resistance to topo IIa inhibitors. This may be true in multiple myeloma where the cells possess a CRM1-mediated mechanism in which topo IIa is exported from the nucleus and away from the DNA, rendering topo IIa inhibitors ineffective to produce cleavable complexes and DNA strand breaks.

In previous reports, our group has shown that myeloma cells, under high-density conditions, will export topo IIa into the cytoplasm both in vivo and in vitro (8–10). We found that nuclear export of topo IIa contributes to drug resistance (9) and that the resistance was not due to differences in drug uptake, cell cycle, or total cellular topo IIa protein levels. In addition, topo IIa nuclear export has been shown to be CRM1 mediated, and topo IIa protein has been found to contain two functional nuclear export signals at amino acids 1,017 to 1,028 and 1,054 to 1,066 (10). Export by both signals was blocked by treatment of the cells with leptomycin B, indicating that a CRM1-dependent pathway mediates export (10). Leptomycin B has been shown to be too toxic for clinical use (12); however, less-toxic CRM1 inhibitors such as ratjadone C and others compounds, as identified in a recent publication, may soon become available (13).

In this study, we showed that myeloma cells grown at high density are highly resistant to topo II–directed chemotherapeutic drugs (Fig. 1A) and that drug resistance correlated with nuclear export of topo IIa (Figs. 1B and 2). Based on these data, we proposed that blocking CRM1-mediated export of topo IIa may make myeloma cells more sensitive to topo II–active agents. To evaluate whether blocking topo IIa export would sensitize cells, we knocked down CRM1 mRNA and protein expression in cells by transfection with CRM1-specific siRNAs and by using the CRM1–inhibiting drug ratjadone C. Ratjadone C was used in this study because it is a potent inhibitor of CRM1, has been shown to have therapeutic value in the treatment of multiple myeloma.

CRM1 inhibition by siRNA and ratjadone C in human myeloma cells was found to prevent nuclear export of topo IIa in plateau-density cell cultures (Figs. 1B and 2). Depletion or inhibition of CRM1 by siRNA or ratjadone C caused high-density myeloma cells to become 4-fold more sensitive to the topo II inhibitors doxorubicin and VP-16 as measured by apoptosis (Fig. 3A–C). Depletion of topo IIa protein by specific topo IIa siRNA knockdown reversed this synergistic effect, indicating that topo IIa was the targeted molecule for CRM1 synergistic activity (Fig. 3C). In addition, we found that blocking CRM1–mediated export sensitized patient myeloma cells obtained from bone marrow aspirates to the topo II poison doxorubicin. Normal peripheral blood mononuclear cells were not sensitized by CRM1 inhibition. It is likely that these cells were not sensitized because they are not replicating at a high rate, unlike the myeloma cells, which double approximately every 24 h. In addition, normal cells do not export topo IIa; therefore, ratjadone C treatment would not affect intracellular localization.

When additional drugs were used in combination with ratjadone C, we found that myeloma cells were sensitized to the topo II inhibitors doxorubicin and VP-16 but not to the alkylating agent melphalan or to the proteasome inhibitor Velcade (Fig. 6).

In conclusion, maintaining topo IIa in the nucleus by inhibition of CRM1 greatly enhanced the cytotoxic effect of the topo II inhibitors doxorubicin and VP-16 in high-density myeloma cells. Band depletion assays indicated that more DNA-topo IIa complexes were stabilized in cells when CRM1 was inhibited (Fig. 5A), and these increased cleavable complexes resulted in increased strand breaks as measured by the comet assay (Fig. 5B) and subsequent apoptosis. These findings may have potential therapeutic value in the treatment of multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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