Proteasomal Degradation of Topoisomerase I Is Preceded by c-Jun NH₂-Terminal Kinase Activation, Fas Up-Regulation, and Poly(ADP-Ribose) Polymerase Cleavage in SN38-Mediated Cytotoxicity against Multiple Myeloma

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ABSTRACT

Topoisomerase I inhibitors are effective anticancer therapies and have shown activity in hematologic malignancies. Here we show for the first time that SN38, the potent active metabolite of irinotecan, induces c-Jun NH₂-terminal kinase activation, Fas up-regulation, and caspase 8-mediated apoptosis in multiple myeloma (MM) cells. Proteasomal degradation of nuclear topoisomerase I has been proposed as a resistance mechanism in solid malignancies. SN38-induced proteasomal degradation of topoisomerase I was observed during SN38-mediated cytotoxicity against MM.1S myeloma cell line but occurred after c-Jun NH₂-terminal kinase activation, Fas up-regulation, and poly(ADP-ribose) polymerase cleavage and failed to protect cells from apoptosis. Differential toxicity was observed against MM cells versus bone marrow stromal cells, and SN38 inhibited adhesion-induced up-regulation of MM cell proliferation when MM cells adhere to bone marrow stromal cells. In addition, SN38 directly inhibited constitutive and inducible interleukin 6 and vascular endothelial growth factor secretion by bone marrow stromal cells. Synergy was observed when SN38 was used in combination with doxorubicin, bortezomib, as well as poly(ADP-ribose) polymerase inhibitor NU1025 and Fas-activator CH11. These findings have clinical significance, because identification of downstream apoptotic signaling after topoisomerase I inhibition will both elucidate mechanisms of resistance and optimize future combination chemotherapy against MM.

INTRODUCTION

Camptothecin and its analogues are major antitumor agents known to cause cellular damage via inhibition of the nuclear enzyme topoisomerase I (1, 2). The toxicities first associated with camptothecin have been considerably reduced in the analogues topotecan and irinotecan. Over the last decade these compounds have been successfully used as anticancer agents in clinic, in particular against solid malignancies, and oral formulations are now available (3). Topoisomerase I inhibitors have also been effective in hematologic malignancies, in particular acute leukemia (4) and non-Hodgkin’s lymphoma (5). There is growing evidence that these compounds may also be useful in the treatment of multiple myeloma (MM). In 1998, the South West Oncology Group reported activity of topotecan in relapsed and refractory MM patients (6). The overall response rate (partial response or better) was 16%, and 46.5% of patients stabilized their disease; only 23.2% of patients progressed on therapy. Significantly, the median progression-free survival was 13 months, and the median survival time was 28 months. By comparison, MM patients treated with bortezomib who achieved a complete, partial, or minimal response had a median progression-free survival of only 12 months and a median survival of 16 months (7).

There have been no prior reports regarding the use of irinotecan in MM. Irinotecan is the produg of a highly potent active topoisomerase I-inhibiting metabolite SN38, which is 1,000-fold more potent than irinotecan in vitro (8). Daily oral doses of 66 mg/m² irinotecan have achieved serum SN38 concentrations of 18.4 nmol/L with a half-life of 16.9 hours (3). Given the previous activity of topotecan against MM, as well as the promising activity of irinotecan against non-Hodgkin’s lymphoma, we investigated the feasibility of irinotecan against MM by conducting a series of experiments using the lactone form of SN38 to assess activity of irinotecan against MM cell lines and patient cells. Using concentrations achievable with a daily oral regimen, we investigated the potency of SN38 against MM patient cells, as well as the effects of SN38 on the bone marrow microenvironment. We also sought to additionally identify the links between DNA damage and cell death, in particular the intracellular signals translating topoisomerase I inhibition into apoptosis in MM. Cellular responses mediating apoptosis include c-Jun NH₂-terminal kinase (JNK) induction, Fas up-regulation, and poly(ADP-ribose) polymerase cleavage via caspase 8. The apoptotic signaling pathways downstream from topoisomerase inhibition may provide avenues for augmentation of the antitumor activity of this class of compounds. For example, targeting the proapoptotic membrane receptor Fas and antiapoptotic nuclear enzyme poly(ADP-ribosyl) polymerase may potentially be used to optimize the clinical use of irinotecan in hematologic malignancies. Proteasomal degradation of topoisomerase I in response to camptothecin treatment has been proposed previously as a resistance mechanism to camptothecins in solid malignancies (9). Therefore, we investigated the possibility of combining bortezomib with irinotecan in MM. In addition, we explored the possible role of proteasome-mediated topoisomerase I degradation as a mechanism of cellular resistance to irinotecan in hematologic malignancies.

MATERIALS AND METHODS

Drugs and Inhibitors. SN38 (Pharmacia, Kalamazoo, MI), bortezomib (Millennium, Cambridge, MA), NU1025, and doxorubicin (Calbiochem, San Diego, CA), were dissolved in DMSO and stored at −20°C, then thawed and diluted in medium for cell culture experiments. Anti-Fas (CH11) was purchased from Cell Signaling (Beverly, MA) and was stored in PBS containing 50% glycerol at −20°C until ready for use. Anti-Fas (human, neutralizing) clone ZB4 was purchased from Upstate Signaling (Chicago, IL), Caspase 8 inhibitor Z-IETD-FMK, caspase 9 inhibitor Z-LEHD-FMK, and JNK inhibitor NU1025 were purchased from Calbiochem.

Multiple Myeloma-Derived Cell Lines and Patient Cells. Dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) human MM cell lines, as well as RPMI-8226 cells resistant to doxorubicin (Dox40) and melphalan (LR5), were cultured as described previously (10). MM patient cells
were obtained from bone marrow samples after informed consent for the use of samples for the purpose of research. The bone marrow mononuclear cells were separated using Ficoll-Hypaque density sedimentation, and plasma cells were purified (>95% CD138+) by positive selection with anti-CD138 magnetic activated cell separation microbeads (Miltenyi, San Diego, CA).

**Bone Marrow Stromal Cell Cultures.** Bone marrow stromal cells were established as described previously (10). Duoset ELISA (R&D System) was used to measure interleukin (IL)-6 and vascular endothelial growth factor (VEGF) in supernatants of 48-hour cultures of bone marrow stromal cells with or without MM.1S cells, in the presence or absence of SN38.

**Growth Inhibition Assay.** MM cells (3 x 10^6 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media, with or without drugs at 37°C. DNA synthesis and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) assays were performed as described previously (10).

**Immunoblotting.** MM.1S cells were cultured with 10 or 100 nmol/L SN38, harvested, washed, and lysed using lysis buffer (radioimmunoprecipitation assay buffer, 2 mol/L Na3VO4, 5 mol/L NaF, 1 mol/L phenylmethyl sulfonyl fluoride, 5 mg/mL leupeptin, and 5 mg/mL aprotinin). Cell lysates were subjected to SDS-PAGE; transferred to polyvinylidine difluoride membrane; and immunoblotted with anti-poly(ADP-ribose) polymerase, anti-caspase 8, anti-caspase 9, and anti-caspase 3 (Cell Signaling, Beverly, MA), as well as anti-JNK and anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA). To measure nuclear topoisomerase I levels, nuclear extracts were carried out using nuclear protein extraction kit (Active Motif, Carlsbad, CA) and immunoassayed with human antitopoisomerase I antibodies (TopoGen, Columbus, OH). Membranes were stripped and reprobed with antimeclonin (Sigma, St. Louis, MO) or antitubulin (Santa Cruz Biotechnology) to ensure equivalent protein loading.

**Topoisomerase I Activity Assay.** Nuclear protein was extracted from MM.1S cells and bone marrow stromal cells, either untreated or treated with SN38 20–100 nmol/L, harvested, washed, and lysed using lysis buffer then purified and hybridized to human U133A microarray chips (Affymetrix, Santa Clara, CA) overnight at 45°C. The chips were then transferred to GenChip fluidics Station 400 (Affymetrix). Phycoryphin-conjugated streptavidin was then bound to make it fluorescent and excitation performed using a confocal laser scanner. Data were analyzed using D-Chip1.3 (11).

**Microarray Analysis.** Briefly, RNA isolation was performed using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. cDNA was synthesized from RNA using a T7 promoter-tailed oligo-dT primer, followed by synthesis of a second strand. Biotinylated cRNA was then purified with microarray chips (Affymetrix, Santa Clara, CA) and immunoblotted with human antitopoisomerase I antibodies (TopoGen, Columbus, OH). Membranes were stripped and reprobed with antimeclonin (Sigma, St. Louis, MO) or antitubulin (Santa Cruz Biotechnology) to ensure equivalent protein loading.

**Topoisomerase I Activity Assay.** Nuclear protein was extracted from MM.1S cells and bone marrow stromal cells, either untreated or treated with SN38 20–100 nmol/L, then purified and hybridized to human U133A microarray chips (Affymetrix, Santa Clara, CA) overnight at 45°C. The chips were then transferred to GenChip fluidics Station 400 (Affymetrix). Phycoryphin-conjugated streptavidin was then bound to make it fluorescent and excitation performed using a confocal laser scanner. Data were analyzed using D-Chip1.3 (11).

**Flow cytometry.** Cell surface staining was performed with FITC- or phycoerythrin-labeled mouse antihuman monoclonal antibodies against CD95 or isotype-matched negative control antibodies (Becton Coulter, Miami, FL). Stained cells were analyzed by a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) using WinMDI software (Scripps Clinic, La Jolla, CA). Ten thousand cells were analyzed and represented in each fluorescent profile.

**Statistical Analysis.** Statistical significance of differences observed in drug-treated versus control cultures was determined using Student’s t test. The minimal level of significance was P < 0.05.

**RESULTS**

**Effect of SN38 on DNA Synthesis of Human Myeloma Cell Lines and Multiple Myeloma Patient Cells.** The effect of SN38 on DNA synthesis in MM cell lines was determined by measuring DNA ([3H]thymidine incorporation during the last 8 hours of 24-hour (cell lines) and 72-hour (patient cells) cultures, in the presence or absence of drug at various concentrations. The absolute counts per minute of cell lines and patient cells are shown (Fig. 1A and D) to illustrate that proliferation of patient cells (Fig. 1D) is less than the proliferation of cell lines (Fig. 1A) and also to illustrate variability in proliferation between cell lines (Fig. 1A). SN38 significantly inhibits DNA ([3H]thymidine incorporation regardless of the baseline uptake of untreated cells. For example, RPMI-8226 cells and derivatives LR5 (resistant to melphalan) and Dox40 (resistant to doxorubicin) all show baseline counts per minute >10^5, well above the baseline cpm of MM.1S and MM.1R cells (<3 x 10^4). The counts per minute were reduced to <20% at 24 hours in all of these lines with <16 nmol/L SN38 (Fig. 1A and B). Inhibition of cell line viability was observed with IC50 <12 nmol/L SN38, as determined by MTS assay at 48 hours (Fig. 1C). The effect of SN38 on DNA synthesis of freshly isolated primary patient MM cells was examined at 72 to 96 hours (Fig. 1D and E). Patient cell baseline DNA thymidine incorporation at 72 hours (<3.5 x 10^5 counts per minute) was below that of human myeloma cell lines but was still markedly inhibited by <10 nmol/L of SN38 (Fig. 1D). The effects of SN38 on patient cell viability at 96 hours are shown in Fig. 1E.

**Effect of SN38 on Patient MM-Derived Bone Marrow Stromal Cells and the Interaction between Bone Marrow Stromal Cells and Multiple Myeloma Cells.** In MM, tumor cells are predominantly localized in the bone marrow microenvironment. In the bone marrow, interactions between MM cells and bone marrow stromal cells trigger the production of cytokines mediating autocrine and paracrine growth and survival of MM cells, as well as protection against drug-induced apoptosis (13). Therefore, we examined the effects of irinotecan on bone marrow stromal cells and MM cells in the bone marrow microenvironment (Fig. 2). Treatment of bone marrow stromal cells with SN38 for 48 hours had a modest effect, reducing viability to 88% at 1 nmol/L and to 85% at 10 nmol/L (P = 0.4). In contrast, MM.1S cell viability was reduced to 18% with 10 nmol/L SN38 for 48 hours (Fig. 2A; P < 0.005). We next examined the effect of SN38 on bone marrow stromal cell-induced MM cell proliferation (Fig. 2B). At 48 hours there was a 2.2-fold increase in the proliferation of MM.1S cells cocultured with bone marrow stromal cells, evidenced by DNA thymidine incorporation (P = 0.01). However, DNA thymidine incorporation by MM.1S cells was reduced to 26% and 6% of control with 1 and 10 nmol/L SN38, respectively (P < 0.003). Therefore, adhesion-induced myeloma cell growth up-regulation was attenuated by SN38. In addition, at 48 hours, SN38 inhibited adhesion-induced up-regulation of interleukin 6 and VEGF secretion in bone marrow stromal cells triggered by coculture with MM.1S cells (Fig. 2C and D). Of additional importance, SN38 also down-regulated constitutive and inducible interleukin 6 and VEGF secretion by bone marrow stromal cells, despite minimal effects on stromal cell viability.

**Apoptotic Signaling Induced by SN38 in MM.1S Cell Lines.** To investigate early death signaling induced by SN38, MM.1S cells were exposed to SN38 for 2 to 24 hours, and cell lysates were examined by Western blot. JNK activation is important in signaling pathways activated by topoisomerase II inhibitor daunorubicin (14), and it has been demonstrated previously that DNA-damaging agents such as 1-beta-D-arabinofuranosylcytosine may induce apoptosis in U937 cells via JNK activation (15). In our current studies, SN38 induced JNK phosphorylation in MM.1S cells as early as 2 hours, and this phosphorylation was sustained exposed to SN38 for 2 to 24 hours, and cell lysates were examined by Western blot. JNK activation is important in signaling pathways activated by topoisomerase II inhibitor daunorubicin (14), and it has been demonstrated previously that DNA-damaging agents such as 1-beta-D-arabinofuranosylcytosine may induce apoptosis in U937 cells via JNK activation (15). In our current studies, SN38 induced JNK phosphorylation in MM.1S cells as early as 2 hours, and this phosphorylation was sustained

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**SN3 IN MULTIPLE MYELOMA**

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as cleavage of caspase 8, caspase 3, and poly(ADP-ribose) polymerase were observed. Although p21 up-regulation was transient, cleavage of caspases and poly(ADP-ribose) polymerase were sustained for at least 24 hours. After 24 hours, significant cleavage of caspase 8 and caspase 3 was in contrast to a lack of caspase 9 cleavage (Fig. 3B, top panel). Rescue from cell death was observed when cells were cocultured with caspase 8 inhibitor Z-IETD-FMK, but there was very little rescue with caspase 9 inhibitor Z-LEHD-FMK (Fig. 3B, bottom panel), indicating that SN38-mediated cytotoxicity occurs predominantly via caspase 8 and caspase 3 in these cells. In addition, rescue of apoptosis was not achieved by the addition of the JNK inhibitor NU1025 (data not shown), indicating that although JNK activation occurs within 2 hours of drug exposure, it is not essential to SN38-induced apoptosis.

**SN38-Induced Apoptosis Is Associated with Up-Regulation of Fas.** JNK activation may result in Fas up-regulation (16), and gene array studies have demonstrated Fas mRNA up-regulation occurs in response to camptothecin (17). Fas activation, which may induce cell death independently of FasL (18), induces caspase 8-mediated apoptosis (19) and is important for etoposide-induced apoptosis (20). We observed SN38-induced up-regulation of surface Fas in MM.1S cells, increasing from 63% at baseline to 81% at 8 hours (Fig. 3C and D). Surface Fas up-regulation was observed as early as 2 hours after exposure to SN38, increased to a peak at 6 to 8 hours, and was sustained for at least 24 hours. Rescue of apoptosis was not achieved by the addition of the anti-Fas inhibitor clone ZB4 (data not shown). Therefore, although Fas up-regulation occurs in response to SN38 treatment, it is not essential to SN38-induced apoptosis. Nevertheless, exogenous activation of Fas may be used to enhance the effects of SN38, as shown below in Fig. 4A.

**Proteasomal Degradation of Topoisomerase I in SN38-Mediated Apoptosis against MM.1S Cells.** Fig. 3E demonstrates that SN38 induced degradation of topoisomerase I between 6 and 8 hours, which was abrogated by the proteasome inhibitor bortezomib, shown in
the bottom panel. Gene array analysis of MM.1S cells treated with 20 nmol/L of bortezomib demonstrated that there was no change in topoisomerase I gene expression after 6-hour treatment with bortezomib (Fig. 3F). Therefore, bortezomib prevented proteasomal degradation of topoisomerase I at a protein level without up-regulation at a gene level and is consistent with data published previously showing that camptothecin-topoisomerase I-cleavable complexes are targets for ubiquitination and proteasomal degradation (21). Therefore, JNK phosphorylation and Fas up-regulation were early events that preceded the SN38-induced proteasomal-mediated topoisomerase I degradation but persisted well after

Fig. 3. SN38 activates caspase-induced apoptosis, JNK phosphorylation, and surface Fas up-regulation, followed by proteasomal degradation of topoisomerase I in MM.1S cells. A. SN38 induced JNK phosphorylation in MM.1S cells as early as 2 hours, which was sustained for at least 24 hours. At 6 hours, up-regulation of p21, as well as cleavage of caspase 8, caspase 3, and PARP, were observed. Although p21 up-regulation was transient, cleavage of caspase 8, caspase 3, and PARP were sustained for at least 24 hours. No significant changes were observed in mcl-1 or bcl-2 during these early time points. B. top, MM.1S cells incubated with 10 and 100 nmol/L SN38 for 24 hours resulted in cleavage of procaspase 8. Only minor cleavage of caspase 9 was observed. FL, full length; CF, cleavage fragment. B, bottom, MTS assay of SN38-treated cells in the presence or absence of caspase 8 inhibitor Z-IETD-FMK or caspase 9 inhibitor Z-LEHD-FMK. C and D. MM.1S cells were treated with SN38 (20 nmol/L) for 2, 4, 6, 8, and 24 hours, labeled with anti-CD95, and examined by flow cytometry for surface expression of Fas. SN38 induced up-regulation of surface Fas expression as early as 2 to 4 hours, shown as increased percentage of cells positive (C) and an increase in the ratio of the mean fluorescence of the sample over mean fluorescence of the background (D). Fas expression increased to a peak at 6 to 8 hours and was sustained for at least 24 hours. E. SN38 induced proteasomal mediated topoisomerase I degradation at between 6 and 8 hours. The top panel demonstrates that SN38 (20 nmol/L) induced degradation of topoisomerase I, but this degradation was prevented by the proteasome inhibitor bortezomib. F. gene array analysis demonstrated that no up-regulation of topoisomerase I occurred at 6 hours, consistent with proteasomal regulation of topoisomerase I at the protein level; bars, ±SEM.

Fig. 4. SN38 is synergistic with Fas activator CH11, PARP inhibitor NU1025, topoisomerase II inhibitor doxorubicin, and bortezomib against MM cells. The inhibitory effects of SN38 (2–4 nmol/L) alone, as well as in combination with (A) CH11 (0.25–0.5 µg/mL), (B) NU1025 (50–100 nmol/L), (C) Dox (50–150 nmol/L), and (D) bortezomib (PS341; 1–2 nmol/L), as measured by MTS assay. Synergy was observed when SN38 was combined with CH11 or NU1025, regardless of the order of drug combination. The addition of Dox to SN38 alone produced a synergistic effect across a range of concentrations, but synergy was not observed when Dox was added first or when both drugs were added simultaneously (data not shown). Synergy was observed when MM.1S cells were exposed to bortezomib (1–2 nmol/L) for 24 hours, then SN38 (2 nmol/L) was added for an additional 24 hours. However, higher concentrations resulted in antagonism. A similar pattern was observed when the drug sequence was reversed. The drug combination data were analyzed according to the Chou-Talalay formula (12) as described in Materials and Methods, and the data are presented in Table 1; bars, ±SEM.
topoisomerase I degradation was maximal. Detection of caspase 8 and caspase 3 cleavage, as well as poly(ADP-ribose) polymerase cleavage, overlapped with proteasomal degradation of topoisomerase I.

Synergistic Anti-Multiple Myeloma Activity among SN38 and Anti-Fas Antibody CH11, Biochemical Poly(ADP-Ribose) Polymerase Inhibitor NU1025, Topoisomerase II Inhibitor Doxorubicin, and Proteasome Inhibitor Bortezomib. Fas activation may result in cell death, and SN38 up-regulates Fas in MM.1S cells. Using MTS assay, we found synergy between the cytotoxicity induced by SN38 and the Fas activating antibody CH11 (Fig. 4A). The combination data were analyzed according to the Chou-Talalay method (12) and presented in Table 1. Topoisomerase I inhibitors cause cell death by damaging DNA, and the enzyme poly(ADP-ribose) polymerase has DNA repair properties (22–24). Therefore, we investigated whether a direct poly(ADP-ribose) polymerase inhibitor could augment SN38-induced cytotoxicity. Fig. 4B shows that the poly(ADP-ribose) polymerase inhibitor NU1025 augments SN38 cytotoxicity as measured by MTS assay; this augmentation was synergistic (Table 1). Fig. 4C shows viability when MM.1S cells were exposed to SN38 (2–4 nmol/L) for 24 hours, with Dox (50–150 nmol/L) added for an additional 24 hours. The addition of low doses of Dox to SN38 produced a synergistic effect across a range of concentrations. However, synergy was not observed when Dox was added first or when both drugs were added simultaneously (data not shown). Synergy was also observed when MM.1S cells were exposed to bortezomib (1–2 nmol/L) for 24 hours, with SN38 (2–4 nmol/L) added for an additional 24 hours (Fig. 4D). However, synergy was not observed at higher concentrations. A similar pattern was observed when the drug sequence was reversed.

Proteasomal Degradation of Topoisomerase I as a Mechanism of Resistance in Myeloma Cell Lines and SUDHL4 Lymphoma Cells. It has been shown previously that the camptothecin-topoisomerase I-cleavable complexes become ubiquitinised and are subsequently degraded by the proteasome (21) and that proteasomal degradation of topoisomerase I may contribute to drug resistance in breast cancer cell lines (9). We have observed that proteasomal degradation of topoisomerase I occurred after apoptotic events have already been initiated in MM.1S cells and failed to protect cells from apoptosis (Fig. 3). We next compared SN38-induced proteasomal degradation of topoisomerase I in MM lines MM.1S (dexamethasone sensitive) with MDR1-expressing RPMI-Dox40 (resistant to doxorubicin) and the bortezomib-resistant B-cell lymphoma cell line SUDHL4 (25). In these three cell lines, treatment with SN38 resulted in inhibition of DNA synthesis, as measured by DNA \[^{3}H\]thymidine incorporation at 24 h (Fig. 5A), confirming that SN38 is not a substrate for MDR1 in this myeloma cell line. However, SUDHL4 cells were resistant to cell death compared with MM cells, as measured by MTS assay at 48 hours (Fig. 5B). Furthermore, poly(ADP-ribose) polymerase cleavage was observed in MM.1S and Dox40 cells at 24 hours but not in SUDHL4 cells (Fig. 5C). SN38 inhibited topoisomerase I at least as efficiently in SUDHL4 as in MM.1S cells, as shown by topoisomerase inhibition assay: topoisomerase I results in relaxation of supercoiled DNA, which can be visualized as slower migration in agarose gel of the relaxed DNA compared with the supercoiled DNA (Fig. 5D). However, treatment with 10 nmol/L SN38 for 24 hours resulted in complete degradation of topoisomerase I in MM.1S cells but not RPMI-Dox40 or SUDHL4 cells (Fig. 5E). There was still a significant level of topoisomerase I in SUDHL4 cells treated with 100 nmol/L of SN38, indicating that proteasomal degradation of topoisomerase I is not a mechanisms of drug resistance in these cells.

DISCUSSION

Topoisomerase I is the cellular target of camptothecin and its derivatives. Camptothecins inhibit topoisomerase I by reversibly stabilizing the covalent topoisomerase I-DNA complex, causing inhibition of DNA re-ligation (1, 2). The camptothecin-topoisomerase I-DNA complexes per se are insufficient to induce a cytotoxic response. Rather, collisions with advancing replication forks convert the drug-stabilized complexes into the irreversible DNA lesions that trigger cell cycle arrest and cell death. Inhibition of DNA synthesis by hydroxyurea (26) and aphidicolin (27) abrogates the cytotoxic action of the drug, demonstrating the S phase specificity of this class of antitumor agents. Furthermore, the induction of DNA strand breaks alone by topoisomerase I and II inhibitors does not necessarily translate into cytotoxicity, and events subsequent to DNA strand breaks are more important than the DNA strand breaks themselves in determining whether apoptosis occurs (28, 29).

We have demonstrated that SN38 at physiologic concentrations in MM.1S cells induced INK activation within 2 hours and cell surface Fas up-regulation that was maximal at 6 to 8 hours, sustained for at least 24 hours, and closely followed by cleavage of procaspase 8 and procaspase 3, as well as poly(ADP-ribose) polymerase. These events were sustained despite proteasomal degradation of topoisomerase I, suggesting that MM.1S cells behaved like type I cells (30). In type I cells, significant activation of caspase 8 generated at the death-inducing signaling complex in the presence of constitutive caspase 3 determines that apoptosis may proceed via a mitochondria-independent pathway. In contrast, type II cells require mitochondrial activation of caspase 9 to activate caspase 8 and caspase 3 during apoptosis. In our current experiments, SN38 triggered Fas up-regulation in MM.1S cells resulting in direct activation of caspase 8 and caspase 3 independently from caspase 9. Importantly, exogenous Fas activation by CH11 in combination with SN38 resulted in synergistic toxicity to MM cells. Downstream from caspase 8, poly(ADP-ribose) polymerase is an important DNA repair enzyme (31), and poly(ADP-ribose) polymerase inhibitors have already shown significant activity in animal models (24). Poly(ADP-ribose) polymerase inhibitors may also augment chemotherapy (24), and there is evidence that poly(ADP-ribose) polymerase inhibitors may be potentially useful in combination with topoisomerase I inhibitors (23). In our current studies we have demonstrated that poly(ADP-ribose) polymerase inhibition by NU1025 can also augment SN38-induced apoptosis, providing the rationale to target poly(ADP-ribose) polymerase to enhance the anti-

Table 1 Inhibitory effects on MM.1S cell growth of SN38 in combination with conventional as well as novel therapies

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<tr>
<th>Compound</th>
<th>FA&lt;sub&gt;Compound&lt;/sub&gt;</th>
<th>FA&lt;sub&gt;[SN38]&lt;/sub&gt;</th>
<th>FA&lt;sub&gt;[Comb]&lt;/sub&gt;</th>
<th>CI</th>
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<tr>
<td>CH11</td>
<td>0.25 0.29 2 0.15 0.51 0.371</td>
<td>0.50 0.30 2 0.15 0.61 0.228</td>
<td>1.00 0.37 6 0.50 0.79 0.375</td>
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<td>NU1025</td>
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<td>100 0.08 2 0.15 0.43 0.365</td>
<td>200 0.10 4 0.26 0.60 0.459</td>
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<tr>
<td></td>
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<tr>
<td>Dox</td>
<td>50 0.07 2 0.15 0.29 0.486</td>
<td>150 0.19 2 0.15 0.41 0.365</td>
<td>50 0.07 4 0.26 0.42 0.459</td>
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<td>150 0.19 4 0.26 0.50 0.465</td>
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<td>PS41</td>
<td>1 0.12 2 0.04 0.34 0.404</td>
<td>2 0.23 2 0.04 0.32 0.818</td>
<td>2 0.27 3 0.06 0.26 1.510</td>
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<td>4 0.36 4 0.06 0.33 1.665</td>
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NOTE: SN38 was tested in combination with four different compounds: CH11 (mg/mL), NU1025 (micromoles), doxorubicin (Dox) (nanomoles), and PS341 (nanomoles). Abbreviations: [Compound], concentration of compound; FA<sup>Compound</sup>, fraction of growth affected by the compound alone; [SN38], concentration of SN38 (nm); FA<sup>[SN38]</sup>, fraction of growth affected by SN38 alone; FA<sup>[Comb]</sup>, fraction growth affected by SN38 and compound in combination; CI, combination index as described in "Statistical Analysis."
tumor activity of DNA damaging agents in general, and irinotecan in particular.

Although the link between DNA damage and cell death has not been well established (14), the intracellular signals translating DNA damage into cell death are being increasingly identified. DNA damaging agents induce the stress-activated protein kinase/JNK pathway, Fas (CD95) expression (32), and subsequent apoptosis in T lymphocytes via the activation of nuclear factor-κB and AP-1 (16). The duration of JNK activation may be important to subsequent apoptosis (33). Cellular responses associated with JNK activation include induction of the c-jun proto-oncogene and other early response genes (34-35). Topoisomerase II inhibitor etoposide induces expression of c-jun in HL-60 myeloid leukemia cells (36), and Fas activation by JNK contributes to etoposide-induced apoptosis in prostate cancer cells (20). Doxorubicin induces apoptosis via the CD95/CD95-L system in human leukemia T-cell lines (37). Fas mRNA up-regulation occurs within hours of treatment with camptothecin-11, as determined by gene array (17). However, although Fas deficiency may contribute to doxorubicin resistance (38), Fas-mediated apoptosis appears to be dependent on efficient caspase activation (39). The Fas-associated death domain can activate caspase 8, and it has been shown previously that caspase 8 activation augments irinotecan-induced apoptosis in prostate cancer cells (40). In our current studies, rescue of apoptosis was achieved by the addition of caspase 8 inhibitor but not of a Fas inhibitor or JNK inhibitor, indicating that although JNK activation and Fas up-regulation occur early during SN38-mediated apoptosis, these events do not mediate SN38-induced cell death. However, activation of Fas augmented SN38-induced cytotoxicity, indicating a potential role for targeting this pathway in combination with irinotecan in chemotherapeutic protocols.

There have been previous reports of sequence-specific synergy between topoisomerase I inhibitors and topoisomerase II inhibitors (41), an additive effect between doxorubicin and SN38 (42), and a contrasting report showing significant antagonism between camptothecin and the topoisomerase II inhibitors etoposide (43) and 4′-(4-acridinylamino)methanesulfon-m-anisidide (26). DNA synthesis is involved in the cytotoxicity of both topoisomerase I and II inhibitors, whereas ongoing RNA transcription may be involved in cytotoxicity of topoisomerase II inhibitors but not topoisomerase I inhibitors (26). The inhibitory effect of camptothecin on RNA transcription may explain the antagonistic effects observed previously between topoisomerase I and II inhibitors and may also explain why in our current studies SN38 down-regulates cytokine secretion by bone marrow stromal cells without affecting cell survival. Against MM cells, we observed synergy when SN38 preceded doxorubicin but not when doxorubicin preceded SN38 or when both were added simultaneously. These data indicate the potential benefit of combining a topoisomerase I inhibitor with a topoisomerase II inhibitor in future clinical trials, but the positive effects of the interactions are not consistent, and careful consideration of dose scheduling would be required.

In our current studies, proteasomal degradation of topoisomerase I was quite variable in both sensitive as well as resistant cells, and the significance of these interactions between the proteasome and the cleavable complex requires additional research. Synergy was observed with bortezomib and irinotecan in MM at extremely low concentrations of SN38 but not at higher concentrations. However, both SN38 and bortezomib display high potency as single agents at higher concentrations in MM, and synergy may not be as important to achieve at these concentrations. Variable patterns of interaction between inhibitors of proteasome and topoisomerase I function have been described previously (44, 45). Bortezomib may enhance the effects of SN38 in DDC841 and WiDr colorectal cells (45), but true
synergy according to the Chou-Talalay (12) criteria has not been demonstrated. Furthermore, SN38 exhibited only weak activity against these cell lines, resulting in only 20% inhibition with 10 to 25 nmol/L after 96-hour incubation and in contrast to the high potency we observed against MM cells. Synergy has also been observed when SN38 was added before the proteasome inhibitor MG-132, but there was antagonism in reverse order (44). The antagonism of MG-132 was attributed to cell cycle arrest that may protect cells from subsequent topoisomerase I inhibitor-induced cell apoptosis. Therefore, close attention to sequence scheduling will be required if both agents are used together in MM.

The high potency of SN38 against MM cells may have clinical implications for drug scheduling. In clinical trials for relapsed refractory non-Hodgkin’s lymphoma, there was no response using 200 mg/m² irinotecan every 3 to 4 weeks, whereas 40 mg/m² daily for 5 days every 3 to 4 weeks produced 17% complete remission and 17% partial remission (46). In addition, 96-hour infusions of 10 mg/m² resulted in no grade 3 or 4 toxicity, whereas 90-minute infusions of 125 mg/m² and 350 mg/m² every 3 weeks resulted in significant toxicity (47–50). These clinical trials suggest that prolonged exposure to low doses of irinotecan may be associated with a significantly higher therapeutic index than short exposure to higher concentrations. Our current data demonstrate the significant activity of SN38 against MM cells at concentrations achievable with daily oral irinotecan, and the use of irinotecan in a low-dose oral schedule to achieve prolonged exposure in patients with MM may, therefore, overcome drug resistance and improve patient outcome.

In conclusion, SN38 in low nanomolar concentrations inhibits proliferation of MM cell lines, including cell lines resistant to dexamethasone, doxorubicin, and melphalan, as well as freshly isolated patient MM cells. Furthermore, by expanding our knowledge of molecular mechanisms of action and resistance to topoisomerase I inhibitors, better combination chemotherapy treatment strategies may be designed. Proapoptotic events induced by SN38 include JNK activation, Fas up-regulation, and cleavage of caspase 8 and poly(ADP-ribose) polymerase, which precede proteasomal degradation of topoisomerase I. Of potential clinical significance, SN38 was synergetic with exogenous Fas activation and poly(ADP-ribose) polymerase inhibition, doxorubicin, and bortezomib. Therefore, clinical trials of irinotecan in MM may be significantly improved in combination with other novel chemotherapy designed to augment the activity of topoisomerase I inhibitors.

REFERENCES


Proteasomal Degradation of Topoisomerase I Is Preceded by c-Jun NH$_2$-Terminal Kinase Activation, Fas Up-Regulation, and Poly(ADP-Ribose) Polymerase Cleavage in SN38-Mediated Cytotoxicity against Multiple Myeloma

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