A Novel Carbohydrate-Based Therapeutic GCS-100 Overcomes Bortezomib Resistance and Enhances Dexamethasone-Induced Apoptosis in Multiple Myeloma Cells

Dharminder Chauhan,1 Guilan Li,1 Klaus Podar,1 Teru Hideshima,1 Paola Neri,1 Deli He,1 Nicholas Mitsiades,1 Paul Richardson,1 Yan Chang,2 Joanne Schindler,2 Bradley Carver,1 and Kenneth C. Anderson1

1The Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School and 2GlycoGenesys, Inc., Boston, Massachusetts

Abstract

Human multiple myeloma is a presently incurable hematologic malignancy, and novel biologically based therapies are urgently needed. GCS-100 is a polysaccharide derived from citrus pectin in clinical development for the treatment of cancer. Here we show that GCS-100 induces apoptosis in various multiple myeloma cell lines, including those resistant to dexamethasone, melphanal, or doxorubicin. Examination of purified patient multiple myeloma cells showed similar results. Specifically, GCS-100 decreases viability of bortezomib/PS-341–resistant multiple myeloma patient cells. Importantly, GCS-100 inhibits multiple myeloma cell growth induced by adhesion to bone marrow stromal cells; overcome the growth advantage conferred by antiapoptotic protein Bcl-2, heat shock protein-27, and nuclear factor-κB; and blocks vascular endothelial growth factor–induced migration of multiple myeloma cells. GCS-100–induced apoptosis is associated with activation of caspase-8 and caspase-3 followed by proteolytic cleavage of poly(ADP-ribose) polymerase enzyme. Combined with dexamethasone, GCS-100 induces additive anti-multiple myeloma cytotoxicity associated with mitochondrial apoptotic signaling via release of cytochrome c and Smac followed by activation of caspase-3. Moreover, GCS-100 + dexamethasone–induced apoptosis in multiple myeloma cells is accompanied by a marked inhibition of an antiapoptotic protein Galectin-3, without significant alteration in Bcl-2 expression. Collectively, these findings provide the framework for clinical evaluation of GCS-100, either alone or in combination with dexamethasone, to inhibit tumor growth, overcome drug resistance, and improve outcome for patients with this universally fatal hematologic malignancy.

Introduction

Multiple myeloma remains fatal despite all available therapies. Initial treatment with dexamethasone effectively induces multiple myeloma cell apoptosis; however, prolonged drug exposures result in the development of chemoresistance (1). The mechanisms mediating drug resistance include defective apoptotic signaling, overexpression of antiapoptotic proteins such as Bcl-2, expression of multidrug resistance (MDR) gene, and the presence of growth-promoting cytokines in the bone marrow microenvironment (2–4). Novel anti-multiple myeloma agents that reverse drug resistance and enhance multiple myeloma cell death are urgently needed.

Glycomics, the study of how a cell’s array of polysaccharides interact with a wide range of proteins and affect cellular phenotype in the context of cell-cell and cell-tissue interactions has identified novel therapeutics (5). Pioneering studies by Raz et al. showed the presence of galactoside-specific binding lectins (Galectins) on the tumor cells, suggesting a potential therapeutic approach targeting carbohydrate-binding protein(s) on the surface of malignant cells (6). Modified citrus pectins (MCP) are complex carbohydrates capable of combining with carbohydrate-binding domain of Galectin-3 (7). These compounds have been shown to inhibit the growth and metastasis of cancer cells and have shown antiangiogenic activity (8). GCS-100 is a MCP in the clinical development. In the present study, we asked (a) whether GCS-100 affects multiple myeloma cell viability and (b) whether a combination of minimally toxic doses of GCS-100 with other conventional anti-multiple myeloma drugs overcomes drug resistance and enhances anti-multiple myeloma activity. We show that (a) GCS-100 induces apoptosis in multiple myeloma cells resistant to conventional and bortezomib therapies, without significantly altering normal cell viability; (b) GCS-100–triggered apoptosis is associated with activation of caspase-8 and caspase-3 followed by proteolytic cleavage of poly(ADP-ribose) polymerase (PARP); (c) GCS-100 overcomes the growth and survival benefits conferred by the bone marrow microenvironment; (d) GCS-100 overcomes drug resistance mechanisms mediated by Bcl-2, heat shock protein-27 (Hsp-27), and nuclear factor-κB (NF-κB); and finally, (e) combination of subtoxic doses of GCS-100 and dexamethasone triggers significant apoptosis in multiple myeloma cells via mitochondria/caspase activation cascades and correlates with marked down-regulation of MCP-binding antiapoptotic protein Galectin-3. These preclinical studies provide the framework for clinical evaluation of GCS-100 either as monotherapy or in combination with less toxic doses of dexamethasone to inhibit multiple myeloma cell growth and overcome drug resistance.

Materials and Methods

Cell culture and reagents. Dexamethasone-sensitive MM.1S and dexamethasone-resistant MM.1R human multiple myeloma cell lines (9, 10) were kindly provided by Dr. Steven Rosen and Nancy Krett (Northwestern University, Chicago, IL). doxorubicin-resistant (Dox-40) and melphanal-resistant (LR-5) RPMI-8226 cells were kindly provided by Dr. William Dalton (Moffit Cancer Center, Tampa, FL). U266, RPMI-8226,
Targeting Carbohydrate-Binding Proteins in Multiple Myeloma

and OCI-M5 multiple myeloma cell lines were obtained from the American Type Culture Collection (Rockville, MD). SUDHL-4 (DHL-4) and SUDHL-6 (DHL-6) lymphoma cell lines were kindly provided by Dr. Margaret Shipp (Dana-Farber Cancer Institute, Boston, MA). Human B-cell lymphoma cell line RC-K8 was kindly provided by Dr. Thomas Gilmore (Boston University, Boston, MA). All cell lines were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM β-mercaptoethanol. Multiple myeloma cells were isolated from patients relapsing after multiple prior therapies including dexamethasone, thalidomide, or bortezomib and purified by CD138-positive selection using CD138 (Syndecan-1) Micro Beads and the Auto MACS magnetic cell sorter (Miltenyi Biotec, Inc., Auburn, CA). An informed consent was obtained from all patients in accordance with the Helsinki protocol. Cells were treated with GCS-100 (GlycoGenesys, Inc., Boston, MA), dexamethasone (Sigma, St. Louis, MO), PK-11195 (Sigma), or bortezomib (Millennium Pharmaceuticals, Cambridge, MA).

Cell viability and quantification of cell death. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International, Inc., Temecula, CA) assay (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (11). Nuclear condensation and segmentation are specific events observed during apoptosis (12, 13). Cell Death Detection ELISA<sup>®</sup> kit was used to quantify cell death, as per manufacturer’s instructions (Roche Applied Sciences, Indianapolis, IN). Apoptosis was also confirmed by Western blot analysis of proteolytic cleavage of PARP enzyme (14). As an additional marker for apoptosis, nuclear morphology was examined using histochemical staining with 4',6-diamidine-2'-phenylindole diHCl (DAPI, Sigma), a fluorescent dye that selectively labels DNA. Briefly, 1.0 × 10<sup>6</sup> MM.1S cells were incubated with GCS-100 (700 μg/ml) for 36 hours, centrifuged onto a glass slide using a cytospin, and fixed in 10% (v/v) formalin in PBS (15 minutes, 23°C). This was followed by incubation with 0.1 μg/ml DAPI in methanol (15 minutes, 23°C). After washing, cells were mounted under a coverslip in glycerol and viewed using a Zeiss Axioplan-2 epifluorescence microscope equipped with UV excitation filter.

Transwell cell migration assay. Cell migration was assayed using a modified Boyden chamber, as previously described (15) using a 24-well plate with 8-μm pore size inserts. Before the assay, the upper and lower chambers were precoated with fibronectin (10 μg/ml). MM.1S cells were starved in 2% serum containing culture medium for 6 hours and then treated with GCS-100 for 4 hours. Multiple myeloma cells (2 × 10<sup>5</sup> cells/ml) were then placed into the upper chamber of the transwell system. To the lower chamber was added RPMI (15% FBS), 50 ng/ml of rVEGF<sub>165</sub> or with GCS-100 (200 or 500 μg/ml). The plates were then incubated at 37°C for 6 hours, and cells in the lower chamber were then harvested. The number of viable migrated cells was counted using a Beckman Coulter counter model ZBII (Beckman Coulter, Fullerton, CA). A migration index was calculated to compare migration of cells relative to control. The migration index was defined as the percentage of live migrated cells in the sample (± drug or ± rVEGF) divided by the percentage of live migrated cells in the control (no drug or rVEGF).

Bone marrow stromal cell cultures. Bone marrow aspirates from patients with multiple myeloma were subjected to mononuclear cell separation by Ficoll-Hipaque density sedimentation and were cultured in vitro to establish long-term bone marrow cultures, as described previously (16, 17). Adherent cell monolayer was harvested in HBSS containing 0.25% trypsin and 0.02% EDTA, washed, and collected by centrifugation.

Effect of GCS-100 on paracrine multiple myeloma cell growth in the bone marrow. To determine the effect of GCS-100 treatment on growth of multiple myeloma cells adherent to bone marrow stromal cells (BMSC), MM.1S cells were cultured for 48 hours in BMSC-coated 96-well plates (Costar, Cambridge, MA) in the presence or absence of various concentrations of GCS-100. After treatment, DNA synthesis was measured by [<sup>3</sup>H]thymidine (Perkin-Elmer, Boston, MA) uptake, as previously described (17). Cells were pulsed with [<sup>3</sup>H]thymidine (0.5 μCi per well) during the last 8 hours of 48-hour cultures. All experiments were done in triplicate.

Measurement of mitochondrial membrane potential (∆Ψ<sub>m</sub> and superoxide generation (O<sub>2</sub>•). Serum starved (2% serum containing culture medium for 6 hours) MM.1S cells were treated with GCS-100 for 24 hours, stained with lipophilic cationic dye CMXRos (MitoTracker Red) in PBS for 20 minutes at 37°C, and analyzed by flow cytometry to assay for alterations in ∆Ψ<sub>m</sub> (18).

Western blotting. Protein lysates were prepared and Western blot analysis was done as previously described (19). Briefly, equal amounts of proteins were resolved by 10% or 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. Filters were blocked by incubation in 5% dry milk in PBST (0.05% Tween 20 in PBS) and probed with anti-cytokyme c, anti-Smac, anti-PAp, Bcl-2 and PARP (BD Biosciences PharmMingen, San Diego, CA); anti-tubulin (Sigma); anti-Galectin-3 (Chemicon International), and cleaved anti-caspase-8, anti-caspase-9, or cleaved anti-caspase-3 (Cell Signaling Technology, Beverly, MA) antibodies. Blots were then developed by enhanced chemiluminesence (Amersham, Arlington Heights, IL).

Isobologram analysis. The interaction between anti-multiple myeloma agents dexamethasone and GCS-100 was analyzed using “Calcusyn” software program (Biosoft, Ferguson, MO and Cambridge, United Kingdom). A combination index (CI) of <1.0 indicates synergism and CI = 1.0 indicates additive activity.

Results

GCS-100 inhibits growth and triggers apoptosis in multiple myeloma cell lines. We first determined whether GCS-100 affects the viability in multiple myeloma cell lines. Treatment of multiple myeloma cell lines (MM.1S, MM.1R, RPMI-8226, LR-5, U266, and Dox-40) with GCS-100 for 24 hours induces a dose-dependent significant (P < 0.005, n = 3) decrease in cell viability in all cell lines (IC<sub>50</sub> range, 350-550 μg/ml; Fig. 1A). To determine whether GCS-100-induced decreased multiple myeloma cell viability is due to apoptosis, various multiple myeloma cell lines were treated at their respective IC<sub>50</sub> for 24 hours, harvested, and analyzed for apoptosis. GCS-100 triggered significant apoptosis in these cells as measured by DNA fragmentation (Fig. 1B), a hallmark of apoptosis (ref. 20; P < 0.004; n = 2). We next examined whether GCS-100 triggers morphologic changes characteristic of apoptosis. Chromatin condensation and nuclear disintegration are typical signs of apoptotic cell death (12, 13). MM.1S cells treated with GCS-100 (700 μg/ml) for 36 hours show a marked increase in nuclear condensation, as indicated by the dense staining pattern of DAPI observed under phase contrast microscopy (Fig. 1C, right). Arrows indicate cell nuclei that are in the process of apoptosis (Fig. 1C, right). In contrast, untreated control cells exhibited homogeneous and intact nuclei (Fig. 1C, left).

Besides nuclear condensation, appearance of apoptotic bodies, and oligonucleosomal DNA, the induction of apoptosis also involves activity of aspartate specific cysteine proteases or caspases (cysteiny1, aspartate-specific proteases), which can either inactivate or activate target substrates such as, PARP by proteolytic cleavage (21). Initiator caspases undergo autocatalytic processing and cleave and activate the downstream executioner caspases that orchestrate cell death (21). Genetic and biochemical evidence indicates that apoptosis proceeds by two major cell death pathways: an intrinsic pathway that involves mitochondrial membrane permeabilization and release of several apoptogenic factors, followed by caspase-9 activation and an extrinsic apoptotic signaling pathway that occurs via caspase-8 activation (22). Both caspase-8 and caspase-9 activate downstream caspase-3.

We next examined whether GCS-100 triggers extrinsic or intrinsic apoptotic signaling pathways. Our results further show that GCS-100 (700 μg/ml) induces activation of caspase-8 and
caspase-9 followed by PARP cleavage (Fig. 1D, top, middle and bottom, respectively). Stress-induced apoptosis correlates with mitochondrial-related events: loss of ΔΨm, and generation of reactive oxygen species (22). No alterations in ΔΨm were observed after treatment of MM.1S cells with GCS-100 at 350 or 700 μg/mL; however, a high dose of GCS-100 (1.4 mg/mL) decreased ΔΨm (Fig. 1E). As a positive control, treatment of MM.1S cells with bortezomib (7 nmol/L) for 24 hours, centrifuged onto a glass slide, fixed with 10% (v/v) formalin in PBS, and stained with DAPI (0.1 μg/mL). Nuclear morphology was examined as described in Materials and Methods. Fluorescence micrographs of DAPI-stained cells from two independent experiments. Arrows indicate apoptotic nuclei with nuclear condensation and DNA apoptotic bodies. D, MM.1S cells were treated with GCS-100 (700 μg/mL) for 48 hours; cytosolic proteins were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with cleaved anti-caspase-8 (top), cleaved anti-caspase-3 (middle), or anti-PARP (bottom) antibodies. Representative of three independent experiments. FL, full-length; CF, cleaved fragment. E, MM.1S cells were treated with the indicated concentrations of GCS-100 for 48 hours, harvested, and analyzed for changes in ΔΨm by CMXRos staining. Columns, means of three independent experiments; bars, ± SD. A as a positive control, cells were also treated with bortezomib (7 mmol/L) for 48 hours and analyzed for ΔΨm. F, MM.1S cells were treated with GCS-100 (700 μg/mL) in the presence or absence of pan caspase (z-VAD-fmk), caspase-8 (IETD-fmk), or caspase-9 inhibitor (LEHD-fmk) for 48 hours and then analyzed for viability using MTT assay. Columns, mean of three independent experiments; bars, ± SD.

We next examined the requirement of caspase activation during GCS-100–induced apoptosis. MM.1S cells were treated with GCS-100 (700 μg/mL) in the presence or absence of caspase-8 inhibitor (IETD-fmk), caspase-9 inhibitor (LEHD-fmk), or pan-caspase inhibitor (z-VAD-fmk). Pan-caspase inhibitor significantly blocked GCS-100–triggered apoptosis; selective caspase-8 inhibition also provided modest rescue from GCS-100–induced apoptosis, whereas blockade of caspase-9 provided no protection against GCS-100–induced apoptosis (Fig. 1F). Of note, however, are the findings that caspase-8 and caspase-3 cleavage are late events occurring at higher doses of GCS-100 and that caspase-8 inhibition does not completely block GCS-100–induced cell death; therefore, it is possible that other cellular death mechanisms besides apoptosis, such as autophagy, may also be active. Our ongoing studies are
focused on examining this issue. Nonetheless, our data suggest that GCS-100 triggers apoptosis in multiple myeloma cells and is associated with caspase-8 mediated apoptotic signaling cascade.

GCS-100 inhibits growth of multiple myeloma patient cells. We next asked whether GCS-100 affects purified patient multiple myeloma cells; tumor cells from six multiple myeloma patients relapsing after multiple prior therapies including dexamethasone, bortezomib, and thalidomide were treated with GCS-100 (100-700 μg/mL) for 24 hours and then analyzed for both viability and apoptosis. Treatment of purified multiple myeloma cells with GCS-100 for 24 hours induces a dose-dependent significant (P < 0.005, n = 3) decrease in cell viability in all patient multiple myeloma cells (IC50 range, 350-500 μg/mL; Fig. 2A). Importantly, multiple myeloma was refractory to bortezomib in four patients and resistant to thalidomide and dexamethasone therapies in two patients. The GCS-100–induced decrease in the viability of patient cells was due to apoptosis in these cells, as evidenced by marked DNA fragmentation and PARP cleavage (Fig. 2B). Taken together, our data shows that GCS-100 effectively inhibits growth of chemoresistant multiple myeloma cells and suggests independent mechanisms of resistance to doxorubicin, melphalan, and dexamethasone versus GCS-100.

GCS-100 does not affect the viability of normal lymphocytes. Normal lymphocytes from five healthy donors were treated with various doses (100-600 μg/mL) of GCS-100 and analyzed for cytotoxicity. In contrast to multiple myeloma cells, survival of normal lymphocytes was not altered significantly (P = 0.27 from j-T trend test) even at higher doses (600 μg/mL) of GCS-100 (Fig. 2C). No significant apoptosis of normal lymphocytes was induced by GCS-100 (data not shown). These findings indicate that GCS-100 has selective anti-multiple myeloma activity.

GCS-100 inhibits paracrine multiple myeloma growth triggered by adherence to bone marrow stromal cells. Our previous studies have shown that bone marrow microenvironment confers cell growth in multiple myeloma cells (1, 2, 16); we therefore next studied the effect of GCS-100 on paracrine multiple myeloma cell growth in the bone marrow milieu. We first examined direct toxicity of GCS-100 on BMSCs from patients (n = 4) using MTT assay, as in our prior studies (23, 24), and observed no significant growth inhibition (5-10%) in response to GCS-100 treatment (data not shown). MM.1S multiple myeloma cells were then cultured with or without BMSCs in the presence or absence of GCS-100. Adherence of tumor cells to BMSCs triggered increased [3H]thymidine uptake of MM.1S cells (1.55-fold increase, P < 0.03); (Fig. 2D), and GCS-100 inhibited this up-regulation of growth in a dose-dependent manner (P < 0.03). These findings show that GCS-100 not only directly targets multiple myeloma cells but also overcomes the cytoprotective effects of the bone marrow microenvironment.

GCS-100 blocks vascular endothelial growth-factor–induced migration of multiple myeloma cells. Vascular endothelial growth factor (VEGF) is elevated in the multiple myeloma bone marrow microenvironment, and our studies showed that VEGF triggers migration and growth of multiple myeloma cells and angiogenesis in multiple myeloma (15). We next therefore examined whether GCS-100 affects VEGF-triggered multiple myeloma cell migration. Multiple myeloma cell migration was evaluated using a modified Boyden chamber. Results show that

Figure 2. A, purified (CD138+) multiple myeloma (MM) cells from patients were treated with indicated concentrations of GCS-100 for 48 hours and analyzed for viability by MTT assay. Points, means of triplicate samples; bars, ±SD. B, purified multiple myeloma cells from patients were treated with GCS-100 for 24 hours and analyzed for apoptosis by DNA fragmentation assay. Columns, means of triplicate samples; bars, ± SD, P < 0.005. Inset, patient multiple myeloma cells were also analyzed for GCS-100–induced apoptosis by proteolytic cleavage of PARP. Representative of independent experiments with similar results from four different patients. C, normal lymphocytes from five healthy donors were treated with indicated concentrations of GCS-100 for 48 hours and analyzed for viability. Points, means of three independent experiments; bars, ± SD. D, GCS-100 inhibits paracrine multiple myeloma cell growth. MM.1S cells were cultured in BMSC-coated or noncoated plates for 48 hours in the presence of medium alone or with the indicated concentrations of GCS-100. DNA synthesis was assessed by [3H]thymidine uptake assay. Columns, means of triplicate cultures; bars, ± SD.
VEGF alone markedly increases MM.1S cell migration and that GCS-100 significantly \( P < 0.05 \) inhibits VEGF-dependent multiple myeloma cell migration (Fig. 3A). Importantly, GCS-100 at the concentrations used in migration assays did not affect survival of multiple myeloma cells (viability >95%), as assessed by MTT assay (data not shown). These findings indicate that GCS-100 may negatively regulate homing of multiple myeloma cells to the bone marrow, as well as their egress into the peripheral blood.

**GCS-100 overcomes Bcl-2–mediated cytotoxicity effects.** Besides the multiple myeloma bone marrow milieu, there are other mechanisms within multiple myeloma cells that facilitate development of drug resistance. For example, Bcl-2 confers resistance to conventional therapies in cancer cells, including multiple myeloma (3). We therefore next examined whether ectopic expression of Bcl-2 in MML1S cells affects responsiveness to GCS-100. MM.1S cells were transfected with either empty vector or vector containing Bcl-2. Western blot analysis show significantly enhanced Bcl-2 protein levels in Bcl-2–transfected MM.1S cells (Fig. 3B, inset). Nontransfected, vector- or Bcl2-transfected MM.1S cells were treated with GCS-100 (700 μg/mL) for 48 hours and then analyzed for alterations in cell viability. GCS-100 significantly decreases cell viability in all cell types \( P < 0.005; \) Fig. 3B). Nonetheless, GCS-100 induced less cell death in Bcl-2–transfected (15 ± 3.3%) compared with empty vector–transfected MM.1S cells. Together, these data show the ability of GCS-100 to overcome the cytotoxic effects of Bcl-2 in multiple myeloma cells.

**GCS-100 triggers apoptosis in cells with mutated IkB-α/activated nuclear factor-κB.** Constitutive activation of the NF-κB signal transduction pathway has been implicated in several hematologic malignancies, including multiple myeloma (2, 25). NF-κB remains inactivated in the cytoplasm due to its complex formation with inhibitory protein IκB-α. Upon growth or survival stimulation, IκB-α is phosphorylated, ubiquitinated, and degraded by proteasomes, leading to the disassociation of p50/65 and its translocation to the nucleus (26). Activated NF-κB binds to the consensus sites present within the promoter region of various growth factors and thereby induces their transcription and secretion (2, 27). Mutations that lead to inactivation of IκB results in constitutively activated NF-κB signaling pathway and growth. RC-K8 lymphoma cell line was recently characterized with mutations that leads to the inactivation of the gene encoding IκB-α resulting in constitutively increased expression of several Rel/NF-κB target genes required for growth and survival (28). We therefore used this cell line as a model to examine whether GCS-100 overcomes the growth conferred by NF-κB activation. As in prior study (28), we observed constitutively activated NF-κB activity without any detectable IκB-α protein in RC-K8 cells (data not shown). Treatment of RC-K8 cells with GCS-100 significantly \( P < 0.005 \) decreases the viability in these cells (Fig. 3C). As a positive control in the assay, GCS-100 triggered marked decrease in the viability of multiple myeloma cells obtained from bortezomib-refractory patients (Fig. 3C). These findings suggest potential utility of GCS-100 to overcome NF-κB–mediated growth, survival, and drug resistance in cancer cells.

**GCS-100 overcomes bortezomib resistance in lymphoma cells.** To exclude the possibility that the ability of GCS-100 to overcome bortezomib resistance is restricted to multiple myeloma cells, we next did similar experiments using bortezomib-resistant SUDHL4 (DHL-4) lymphoma cells (11). DHL4 cells were treated with various concentrations of GCS-100 for 24 and 48 hours, harvested, and analyzed for cell viability. GCS-100 decreased viability of DHL4 cells, with an IC50 of 300 to 350 μg/mL at 48 hours (Fig. 3D). Together, these findings show that GCS-100 overcomes bortezomib resistance in multiple myeloma as well as other cancer cell types, and suggest its potential clinical use in bortezomib-refractory cancer patients.

**Combined treatment with GCS-100 and antagonist to peripheral benzodiazepine receptor PK-11195 or dexamethasone induces synergistic/additive anti-multiple myeloma activity.** Having shown that GCS-100 is an effective anti-multiple myeloma agent, we next examined whether GCS-100 can be combined with other anti-multiple myeloma agents to enhance cytotoxicity. The rationale for combining the two agents is provided, in part, by the mechanisms of their action. For example, our findings show that GCS-100 primarily triggers caspase-8 mediated apoptotic signaling cascade (extrinsic pathway); therefore, we asked whether combining GCS-100 with another therapeutic agent that specifically targets mitochondria (intrinsic pathway) may increase overall anti-multiple myeloma activity. To address this issue, we used PK-11195, an antagonist to mitochondrial peripheral benzodiazepine receptor (29). Treatment of cells with GCS-100 + PK-11195 triggered synergistic anti-multiple myeloma activity, as evidenced by a significant decrease in the viability of MM.1S cells (Fig. 4A).

To further address this issue, we combined GCS-100 with dexamethasone, an agent commonly used to treat multiple myeloma that induces mitochondrial apoptotic signaling (19). MM.1S cells were treated with dexamethasone (0.025 μmol/L) and indicated concentrations of GCS-100 and analyzed for viability. GCS-100 significantly enhances the anti-multiple myeloma activity of dexamethasone induced (additive effect with CI = 1.0; Fig. 4B). Similar results were obtained using purified patient multiple myeloma cells, without any significant effect of combined low doses of these agents on the viability of normal lymphocytes (data not shown). Furthermore, combined low doses of GCS-100 + dexamethasone triggered PARP cleavage and activation of caspase-3 (Fig. 4C), whereas neither agent alone at these low doses affected PARP or caspase-3 cleavage (Fig. 4C). We next determined whether low doses of GCS-100 + dexamethasone trigger release of mitochondrial proapoptotic proteins Smac and cytochrome c. MM.1S cells were treated with GCS-100 (125 μg/mL), dexamethasone (0.025 μmol/L), or GCS-100 + dexamethasone; cytosolic extracts were then prepared and subjected to immunoblot analysis with anti-Smac or anti-cytochrome c antibodies (Fig. A, top and middle). Treatment of MM.1S cells with GCS-100 + dexamethasone induces the release of both cytochrome c and Smac from mitochondria to cytosol (Fig. 4D, top and middle). In contrast, neither agent alone at these concentrations induced significant release of cytochrome c and Smac. Reproducing the immunoblots with anti-tubulin antibodies confirms equal protein loading (Fig. A, bottom).

**GCS-100 + dexamethasone–induced apoptosis in multiple myeloma cells is associated with down-regulation of an antiapoptotic protein Galectin-3.** Previous studies have linked MCP to Galectin-3, a carbohydrate-binding protein (7, 30). Galectin-3 shares an affinity for β-galactoside-containing glycoconjugates and a conserved sequence of the sugar-binding motif (31, 32). Importantly, Galectin-3 is associated with tumor cell adhesion, proliferation, differentiation, angiogenesis, and metastasis (31). Other studies showed that Galectin-3 is an antiapoptotic protein (33–35) and shares a functional BH1 (NWGR) domain of Bcl-2 family (36). In the context of multiple myeloma, Bcl-2 is
known to confer drug resistance; however, the expression and function of Galectin-3 in multiple myeloma cells is unclear. We therefore first examined the expression of Galectin-3 in various multiple myeloma cell lines. Western blot analysis shows that Galectin-3 is differentially expressed in various multiple myeloma cell lines (Fig. 5A and B, top). Reprobing the blots with control protein tubulin confirmed equal protein loading in each lane (Fig. 5A and B, bottom). These results are consistent with other studies showing Galectin-3 expression in two multiple myeloma cell lines AF10 and H929 (37).

Galectin-3 has been reported as a negative regulator of apoptosis (34, 36, 38). Because both Bcl-2 and Galectin-3 are antiapoptotic proteins, we asked whether GCS-100-, dexamethasone-, or GCS-100 + dexamethasone–triggered multiple myeloma cell death modulates their expression. Treatment of MM.1S cells with either GCS-100 or dexamethasone does not alter either Galectin-3 or Bcl-2 expression (data not shown). Importantly, combined treatment of multiple myeloma cells with subtoxic concentrations of GCS-100 and dexamethasone markedly decreases Galectin-3 expression (Fig. 5C, top), without any changes in tubulin (control protein; Fig. 5C, bottom). Furthermore, GCS-100 + dexamethasone does not affect Bcl-2 levels (Fig. 5D, top), suggesting that alterations in Galectin-3 are specific. Prior studies have shown that Galectin-3 inhibits a major mitochondrial proapoptotic protein cytochrome c, and our results show that GCS-100 + dexamethasone triggers the release of cytochrome c from mitochondria to cytosol. Together, these results suggest that GCS-100 + dexamethasone–induced multiple myeloma cell apoptosis likely proceeds by eliminating the inhibitory effect of Galectin-3 on cytochrome c.

**Discussion**

Our present study shows the following: (a) GCS-100, a novel carbohydrate-based agent, induces apoptosis in multiple myeloma cells resistant to conventional and bortezomib therapies without affecting normal lymphocyte viability; (b) GCS-100 inhibits growth of multiple myeloma cells even in the presence of BMSCs; (c) GCS-100 blocks VEGF-induced migration of multiple myeloma cells, suggesting its antiangiogenic activity; (d) GCS-100 overcomes both the growth/survival advantage conferred by NF-κB and the cytoprotective effects of antiapoptotic protein BCL-2; (e) GCS-100–induced apoptosis occurs predominantly via caspase-8-to-caspase-3 signaling pathway, whereas GCS-100 does not significantly alter mitochondrial apoptotic signaling, including alterations in ΔΨm, O2 production, or activation of caspase-9; (f) the combination of low-dose GCS-100 and PK-11195 triggers synergistic anti-multiple myeloma activity; and finally, (g) the combination of low dose GCS-100 and dexamethasone triggers additive anti-multiple myeloma activity via both caspase cascade as well as inhibition of an antiapoptotic protein Galectin-3.

The finding that GCS-100 induces apoptosis in multiple myeloma cell lines and patient cells is consistent with various other studies showing the antitumorigenic activity of MCP both in vitro and in vivo (7, 8, 39, 40). The mechanistic studies show that GCS-100–induced apoptosis is associated with activation of classic extrinsic cell death signaling pathway caspase-8/caspase-3/ PARP. Conversely, inhibition of caspase-8 but not caspase-9 significantly attenuates GCS-100–triggered cell death. Because GCS-100 targets cell surface carbohydrate-binding proteins, it is likely to first affect cell surface receptors (i.e., activation of death receptors), which are
well known to trigger downstream caspase-8 via FADD (3, 41). Of note, however, is the finding that caspase-8/caspase-3 cleavage is late event occurring at higher doses of GCS-100 and that caspase-8 or pan-caspase inhibition does not completely block GCS-100–induced cell death. These data suggest that other nonapoptotic form of cell death, such as autophagy, may also be active, and our ongoing studies are focusing on this issue. Nevertheless, the present study shows that GCS-100 has marked antitumor activity and is a novel therapeutic agent in multiple myeloma. Importantly, GCS-100 is less toxic to normal cells and therefore has therapeutic index suggesting a favorable side effect profile.

Initial studies by Raz et al. showed that tumor cell surface lectins play an important role in cell-to-cell and cell-to-substratum interactions during metastasis (42); therefore, lectins are attractive therapeutic targets in cancer. In this context, adhesion of multiple myeloma cell to BMSCs triggers growth and development of drug resistance (43). Our data shows that GCS-100, which targets cell surface lectins, markedly abrogates both multiple myeloma cell growth in the presence of BMSCs and VEGF-induced migration of multiple myeloma cells, suggesting that GCS-100 causes disruption of multiple myeloma-to-BMSCS interactions, thereby blocking the tumor cell growth and survival signaling conferred by bone marrow milieu.

We and others have shown intrinsic signaling cascades that mediate drug resistance in multiple myeloma cells, including Bcl-2 (1), Hsp-27, or constitutive activity of NF-κB (11, 44). Our data suggest that Bcl-2 is unlikely to block GCS-100–induced cytotoxicity. First, Bcl-2 is predominantly localized in mitochondria and modulates the caspase-9 signaling pathway via alterations in the mitochondrial membrane potential/cytochrome c release (22). In contrast, GCS-100 does not affect either of these signaling pathways. Second, GCS-100 decreases viability of MM.1S cells with ectopically overexpressed Bcl-2. A residual resistance to GCS-100 was noted in Bcl-2–transfected cells, which may be due to cytoplasmic sequestration of Bcl-2 with other anti-death proteins. Nevertheless, our data show the ability of GCS-100 to overcome the cytotoxic effects of Bcl-2 in multiple myeloma cells.

Hsp-27, like Bcl-2, contributes to the development of drug-resistance. Elevated levels of Hsp-27 transcripts have been observed in multiple myeloma versus normal cells (45). Our study using oligonucleotide arrays showed that Hsp-27 mRNA and protein is highly expressed in dexamethasone-resistant multiple myeloma cells compared with dexamethasone-sensitive multiple myeloma cells (44, 46). Importantly, GCS-100 induces apoptosis in both cell types in a similar time- and dose-dependent manner. Furthermore, Hsp-27 confers resistance to proteasome inhibitor PS-341/bortezomib in DHL-4 lymphoma cells (11), and our present study shows that treatment of DHL-4 cells with GCS-100 markedly decreases the viability in these cells. Together, these data suggest that GCS-100 overcomes Hsp-27–mediated drug resistance.

Recent studies have reported that Galectin-3, like Bcl-2, is an antiapoptotic molecule (34, 36, 38) which is linked to MCP (31, 47). Our data show that GCS-100 does not alter Galectin-3 expression; however, GCS-100 + dexamethasone down-regulates Galectin-3 expression. The finding that GCS-100 alone does not change Galectin-3 is likely consistent with the recent report showing that Galectin-3 translocates from nucleus to cytoplasm during apoptosis without changes in its total expression level. It is also possible that Galectin-3 is differentially regulated in response to various apoptotic stimuli in distinct cell types. Our ongoing studies are therefore focusing on these issues using stable overexpression of Galectin-3 or its mutants in multiple myeloma cells. Importantly, GCS-100 + dexamethasone–induced apoptosis in multiple myeloma cells is associated with decreases in Galectin-3; because Galectin-3 is an antiapoptotic protein (34, 36, 38), its down-regulation may mediate or enhance apoptosis. Our finding that GCS-100 + dexamethasone negatively regulate Galectin-3 expression is consistent

Figure 4. A. MM.1S cells were treated with the indicated concentrations of GCS-100, PK-11195, or GCS-100 + PK-11195 for 24 hours, harvested, and analyzed for viability by MTT assay. B, MM.1S cells were treated with the indicated concentrations of GCS-100 alone (–, –) or together with dexamethasone (Dex; 0.025 μmol/L, –) for 24 hours, harvested, and analyzed for viability by MTT assay. Columns, means from three independent experiments; bars, ± SD. C, MM.1S cells were treated with GCS-100 (125 μg/mL), dexamethasone (0.025 μmol/L), or GCS-100 (125 μg/mL) + dexamethasone (0.025 μmol/L) for 24 hours and harvested. Total protein lysates were subjected to immunoblot analysis using anti-PARP (top), cleaved caspase-3 (middle), or tubulin (bottom) antibodies. FL, full-length; CF, cleaved fragment. D, MM.1S cells were treated with GCS-100 (125 μg/mL), dexamethasone (0.025 μmol/L), or GCS-100 + dexamethasone for 24 hours and harvested. Cytosolic proteins were subjected to immunoblot analysis using anti-Smac (top), cytochrome c (middle), or tubulin (bottom) antibodies. Representative of three independent experiments with similar results.
with this later hypothesis, resulting in increased overall antitumor activity. Furthermore, Galectin-3 is known to inhibit a major mitochondrial proapoptotic protein cytochrome c (35), and our results show that GCS-100 + dexamethasone triggers the release of cytochrome c from mitochondria to cytosol. Together, these results suggest that dexamethasone triggers caspase-9 (19), coupled with our results that GCS-100 induce caspase-8, suggests that combined treatment of multiple myeloma cells with GCS-100 and dexamethasone induces both intrinsic (mitochondrial/caspase-9) and extrinsic (caspase-8 mediated) apoptotic signaling cascades. Importantly, combination therapy with GCS-100 and dexamethasone therefore may (a) allow use of subtoxic concentrations of each agent, (b) delay or prevent development of drug resistance, and (c) permit escalating additive doses of these agents to increase the apoptotic threshold.

Ongoing studies are evaluating the antitumor activity of GCS-100, either alone or in combination with conventional agent dexamethasone, using our multiple myeloma animal models (48, 49). Collectively, these findings provide the framework for clinical trials of GCS-100, either alone or in combination with dexamethasone, to enhance clinical efficacy, reduce toxicity, and overcome drug resistance to conventional and bortezomib therapy in patients with relapsed/refractory multiple myeloma.

Acknowledgments

Received 1/17/2005; revised 6/7/2005; accepted 6/16/2005.

Grant support: NIH grants CA 50947, CA 78373, and CA100707; Doris Duke Distinguished Clinical Research Scientist Award (K.C. Anderson); Multiple Myeloma Research Foundation Senior Research Award (D. Chauhan); Myeloma Research Fund; and Cure Myeloma Fund.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Figure 5. A and B, expression of Galectin-3 in multiple myeloma and lymphoma cells. Immunoblot analysis of Galectin-3 was done using 40 μg of cell lysates from MM.1S, MM.1R, U266, Dox-6, RPMI-8226, OCI-My5, S6B45, DHL-4, DHL-6, patient multiple myeloma (CD138+) cells. Protein lysates from BT549-Galectin-3 overexpressing cells and human recombinant Galectin-3 protein were used as positive control for Galectin-3 expression. C, MM.1S cells were treated with GCS-100 (125 μg/mL) + dexamethasone (Dex, 0.025 μmol/L) for 48 hours and harvested. Protein lysates were subjected to immunoblot analysis using anti-Galectin-3 (top) or tubulin (bottom) antibodies. Representative of two independent experiments with similar results. D, MM.1S cells were treated with GCS-100 (125 μg/mL) + dexamethasone (0.025 μmol/L) for 48 hours and harvested. Protein lysates were subjected to immunoblot analysis using anti-Bcl-2 (top) or tubulin (bottom) antibodies. Representative of two independent experiments.

References

A Novel Carbohydrate-Based Therapeutic GCS-100 Overcomes Bortezomib Resistance and Enhances Dexamethasone-Induced Apoptosis in Multiple Myeloma Cells

Dharminder Chauhan, Guilan Li, Klaus Podar, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/18/8350

Cited articles
This article cites 48 articles, 27 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/18/8350.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/65/18/8350.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.