GCS-100, a novel galectin-3 antagonist, modulates MCL-1, NOXA, and cell cycle to induce myeloma cell death

Matthew J. Streetly,1,2 Lenushka Maharaj,3 Simon Joel,3 Steve A. Schey,4 John G. Gribben,3 and Finbarr E. Cotter3

1Department of Haematology, Guys Hospital, Guy’s and St Thomas’ Foundation Trust, London; 2Centre for Experimental Cancer Medicine, Barts and the London School of Medicine, London; and 3Department of Haematology, Kings College Hospital Foundation Trust, London, United Kingdom

GCS-100 is a galectin-3 antagonist with an acceptable human safety profile that has been demonstrated to have an antymyeloma effect in the context of bortezomib resistance. In the present study, the mechanisms of action of GCS-100 are elucidated in myeloma cell lines and primary tumor cells. GCS-100 induced inhibition of proliferation, accumulation of cells in sub-G1 and G2 phases, and apoptosis with activation of both caspase-8 and -9 pathways. Dose- and time-dependent decreases in MCL-1 and BCL-XL levels also occurred, accompanied by a rapid induction of NOXA protein, whereas BCL-2, BAX, BAK, BIM, BAD, BID, and PUMA remained unchanged. The cell-cycle inhibitor p21G1p was up-regulated by GCS-100, whereas the procycloprotein CYCLIN E2, CYCLIN D2, and CDK6 were all reduced. Reduction in signal transduction was associated with lower levels of activated IκBα, IκB kinase, and AKT as well as lack of IκBα and AKT activation after appropriate cytokine stimulation (insulin-like growth factor-1, tumor necrosis factor-α). Primary myeloma cells showed a direct reduction in proliferation and viability. These data demonstrate that the novel therapeutic molecule, GCS-100, is a potent modifier of myeloma cell biology targeting apoptosis, cell cycle, and intracellular signaling and has potential for myeloma therapy. (Blood. 2010;115(19):3939-3948)

Introduction

Over the past decade, advances in the understanding of myeloma biology and the microenvironment have translated into improved outcomes with novel therapeutic agents.1,2 Apoptosis, cell cycle, PI3K/AKT, and angiogenesis pathways are all deregulated and lend themselves to therapeutic targeting.2 Antiapoptotic proteins MCL-1, BCL-2, and BCL-XL are commonly increased,3 and microenvironment factors, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor, contribute to oncogenesis via these pathways.3 Conversely, disruption of signaling pathways leading to a decrease in MCL-1/BCL-XL expression is associated with increased apoptosis5,6 via the mitochondrial/intracellular pathway, emphasizing the pivotal role for these antiapoptotic molecules in myeloma cell survival.7,8 Disruption of normal proliferation and cell-cycle control are additional factors in myeloma cell survival9 with constitutive expression of PI3K/AKT observed in many myeloma cell lines.10-12 AKT activation is enhanced by multiple cytokines within the bone marrow4,13-15 and is associated with cell proliferation,13,15-17 cell survival,11,12,14 adhesion,18 and migration.19 AKT-mediated phosphorylation of forkhead transcription factor and glycogen synthase kinase 3β (GSK3β) leads to their inactivation with subsequent down-regulation of p27Kip1, up-regulation of cyclin D1, and ultimately G0/S phase progression. Inhibition of PI3K reverses this process and leads to G1 cell-cycle arrest.15,16 In addition, the proapoptotic protein BAD is activated by AKT13 and mediates insulin-like growth factor-1 (IGF-1)-associated tumor necrosis factor-related apoptosis-inducing ligand insensitivity. Activation of AKT by IGF-1 stimulates nuclear factor-κB (NF-κB) and subsequently up-regulation of FLIP (caspase-8 inhibitor), XIAP (caspase-9 inhibitor), and survivin (caspase-3 inhibitor),11 resulting in increased apoptosis resistance. Drugs targeting this process reduce myeloma cell proliferation and survival.18 Galectin-3 is expressed in myeloma cells and is 1 of a group of 14 lectins that bind β-galactoside-containing carbohydrates, via a carbohydrate recognition domain.20,21 It is predominantly localized in the cytoplasm where it acts to suppress apoptosis but may translocate to the nucleus22 and/or secrete from the cytoplasm where it then has proapoptotic effects.23 Galectin-3 appears important in several malignantities, including B-cell lymphomas, with a role in angiogenesis and metastases.20,21,23 There is specific sequence homology to the BH-1 domain of BCL-224 within the carbohydrate recognition domain of galectin-3 containing the NWGR motif critical to the formation of BCL-2 dimers.25 Galectin-3/BCL-2 heterodimerization enhances the BCL-2 antiapoptotic effect.26 Little, however, is known about the role of galectin-3 in multiple myeloma. An observational study of bone marrow samples detected galectin-3 expression in 25% of 16 samples studied.21 A second study observed the expression of galectin-3 in 8 cell lines27 and further confirmed this molecule as a potential target for myeloma therapy.

GCS-100, a modified citrus pectin carbohydrate, binds to galectin-3. Activity has been observed in several animal tumor models28-30 and phase 1 studies in solid tumors31 and CLL32,33 with minimal toxicity to normal lymphocytes22 or myelopoiesis.33 Antimyeloma activity for GCS-100 has previously been reported with synergy in combination with dexamethasone, bortezomib, or PK11195.27
This study examines the effects of GCS-100 as a promoter of apoptosis in myeloma cells.

Methods

Reagents

Recombinant human IGF-1, recombinant human IL-6, and recombinant human TNF-α were purchased from PeproTech EC. Z-VAD-fmk was purchased from Promega. GCS-100 was supplied by Prospect Therapeutics. Antibodies to the following proteins were used: galectin 3 (clone 3-194804) and BID (R&D Systems), caspase-8 (3-1-9), MCL-1, (22) BCL-2X (2H12; all BD Biosciences PharMingen), BAX (2D2), BCL-2, CDK2 (D112), and MCL-1 (S-19; Santa Cruz Biotechnology), PUMA and BAK (Abcam), BIM (Chemicon), NOXA (1143071; Alexis Biochemicals), caspase-9 (5B4) and caspase-3 (both Stressgen), BAD, Cyclin D2, Cyclin E2, CDK6 (DCS83), CDK4 (DCS156), p15INK4b, p16INK4a, p21cip1 (DCS60), p27kip1, IκBα (112B2), phospho-IκBα (ser32/36; 5A5), IκB kinase α (IκKα), phospho-IκKα (ser180), phospho-NF-κBp65 (ser536; 7F1), phospho-AKT (ser473), and phospho-STAT3 (Tyr705; 3E2; all Cell Signaling), β-actin (clone AC-74; Sigma-Aldrich), and goat anti–mouse–conjugated horseradish peroxidase and goat anti–rabbit–conjugated horseradish peroxidase (both BD Biosciences PharMingen). All other materials were supplied by Sigma-Aldrich unless stated.

Cell culture

Myeloma cell lines U266 and RPMI 8226 were purchased from ECACC. OPM-2 cells were a kind gift from Professor T. Lapin (Belfast, United Kingdom). Cell lines were cultured in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (all PAA Laboratories), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator with 5% CO2 at 37°C. Myeloma cell lines U266 and RPMI 8226 were purchased from ECACC. Cell line RPMI 8226 cells were cultured in 96-well plates at a final density of 10^5 cells/mL in 10% FBS and incubated at 37°C for 72 h. Cells were washed in PBS, and resuspended in 70% ethanol.

Cell-cycle assessment

Cells were harvested, washed in PBS, and lysed in an equal volume of lysis buffer (50 mM Tris base, pH 7.4, 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, 1% NP40, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 2% protease inhibitor cocktail [protinprot, leupeptin, bestatin, pepstatin-A, E-64, and 4-2-aminoethylbenzenesulfonyl fluoride]) on ice for 30 minutes. The solution was cleared by centrifugation at 10,000 rpm for 20 minutes at 4°C. Protein quantitation was determined using the Bradford method (Bio-Rad Lab). Equal protein concentrations were loaded in denaturing sample buffer (125 mM Tris-HCl, pH 6.8, glycerol 10%, sodium dodecyl sulfate [SDS] 2%, 2-mercaptoethanol 5%), separated by 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE), and electrottransferred to Immobilon P polyvinylidene difluoride (PVDF) membrane. The membranes were blocked for 1 hour in 5% dried milk in Tris-buffered saline with Tween-20 (TBST; 20 mM Tris base, pH 7.5, 150 mM NaCl, 0.5% Tween-20) or 5% bovine serum albumin/TBST (for phosphor-specific antibodies). Membranes were incubated overnight at 4°C, washed for 30 minutes in TBST, followed by incubation for 1 hour with secondary antibody. After a final washing step with TBST for 30 minutes, then TBS (20 mM Tris base, pH 7.5, 150 mM NaCl) for 20 minutes, bound antibody was visualized using Supersignal West Pico Chemiluminescent ECL (Perbio Science UK). Results shown are representative of a minimum of 2 independent experiments.

Primary cell culture, viability assay, and stromal cell coculture

Primary plasma cells were obtained from bone marrow aspirate samples from patients with relapsed myeloma after ethical approval from Barts and The London School of Medicine and written informed consent. All samples were collected adhering to Good Clinical Practice and the Helsinki Protocol.

Primary samples were collected into ethylenediaminetetraacetic acid, mixed 1:1 with RPMI 1640, and enriched for CD138-positive cells by negative selection (antiglycophorin A, CD2, CD21, CD33, CD41, CD45RA, CD66b) using 50 μL/mL RosetteSep MM-enrichment cocktail (Stem Cell Technologies) at room temperature for 20 minutes following the manufacturer’s instructions. Mononuclear cells were separated by Ficoll-Hypaque (NYcomed) and yielded more than 95% plasma cell purity.

Primary plasma cells were cultured in RPMI 1640 with 10% FBS and 2% bovine serum albumin and incubated at 37°C for 72 hours. To assess the effect of GCS-100 on primary cell viability and proliferation, 1 × 10^5 cells/mL were cultured in 96-well plates for 48 hours and then incubated with GCS-100 (0, 350, 500, or 700 μg/mL) for 48 hours. Cell viability and cell number were assessed using the MTS Proliferation (Guava Technologies) by adding Viacount Flex reagent as per the manufacturer’s instructions. A MTS-96 automated cell analyzer with CytoSoft analysis software was used (both Guava Technologies).

The effect of GCS-100 was also assessed by the ViaCount assay on U266 cells and the human stromal cell line HS-5.

The influence of coculture with stromal cells on GCS-100 activity was assessed. HS-5 stromal cells were plated to a final density of 2.5 × 10^4 cells/mL in 96-well plates and allowed to adhere overnight. The following
day, 1 x 10^5 primary myeloma cells/mL were layered over the HS-5 stroma, and recombinant human interleukin-6 (R&D Systems) was added at a final concentration of 10 ng/mL. After a 48-hour culture, GCS-100 effects on cell viability and cell number were measured as described.

Statistical analysis

The Student paired t test was used to compare quantitative changes observed from a minimum of 2 independent experiments. A P value less than or equal to .05 was considered statistically significant.

Results

GCS-100 inhibits myeloma cell growth, induces apoptosis, and is associated with accumulation of cells in sub-G1, with corresponding loss of cells in S and G2/M phase

A dose-dependent reduction of proliferation was confirmed for RPMI 8226, U266, and OPM-2 cells incubated with GCS-100 (0-1500 µg/mL) for 48 hours with growth inhibition assessed using the Alamar Blue assay. RPMI 8226 and OPM-2 cells were markedly more sensitive than U266 cells (half-maximal effective concentration: 498, 401, and 737 µg/mL, respectively; Figure 1A).

A significant dose-dependent induction of apoptosis occurred in both RPMI 8226 and U266 cells, with the mean proportion of annexin V–positive RPMI 8226 cells increasing from 14% in control cells to 25% (P < .001), 41% and 58% (both P < .001) when incubated with 250 µg/mL, 500 µg/mL, or 800 µg/mL GCS-100, respectively (Figure 1B). U266 cells also underwent apoptosis but required higher concentrations of GCS-100 (annexin V–positive control cells, 11.5%; 500 µg/mL, 40%, P < .001; and 800 µg/mL, 57%, P < .001; Figure 2A). The effect on Δψm for U266 cells was less pronounced; however, the proportion of cells with low Δψm uptake increased from 14% in control cells to 34% in those exposed to 800 µg/mL of GCS-100 (P = .02; Figure 2A). Characterizing the effect of GCS-100 specifically on the mitochondria, the proportion of cells with low Δψm (3) uptake was examined in cells that excluded the viability dye DAPI, thereby ensuring that only cells with an intact cell membrane were examined. RPMI 8226 cells incubated with 250 µg/mL or greater for 48 hours had a significant fall in Δψm. The proportion of DiOC6(3) low/DAPI-negative cells from 11% at baseline to 15% (P = .02) and 30% (P < .001) when treated with 500 µg/mL and 800 µg/mL GCS-100, respectively (Figure 2B). The effect of GCS-100 on RPMI 8226 and U266 cells was also examined over time (Figure 2C). A significant increase in DiOC6(3) low cells was detected after 24 hours of incubation with 500 µg/mL or 800 µg/mL GCS-100 in RPMI 8226 (P < .001) or U266 (P ≤ .04) cells, respectively (Figure 2C), confirming both a time- and dose-dependent effect on transmembrane mitochondrial potential by GCS-100.

Loss of Δψm is associated with mitochondrial/caspase-9 apoptosis. Further characterization of cell death in RPMI 8226 cells by detection of caspase-9, -8, and -3 activation was assessed by Western blot analysis (Figure 2D). Caspase-8 and caspase-9 cleavage was observed after 4 hours of exposure to 500 µg/mL GCS-100, and activation of caspase-3 was observed after 24-hour GCS-100 exposure, suggesting that the intrinsic and extrinsic apoptosis pathways are activated by GCS-100–induced apoptotic cell death.

GCS-100 reduces prosurvival proteins MCL-1 and BCL-XL

The BCL-2 family protein MCL-1 has been shown to be essential to the survival of myeloma cells.7,8 Western blot analysis of RPMI 8226 cells exposed to GCS-100 (500 µg/mL) demonstrated a reduction of both MCL-1 and BCL-XL levels after 24 hours of exposure (Figure 3A). Levels of BCL-2 and galectin-3 expression, as expected, were not altered. MCL-1 reduction in RPMI 8226 cells was concentration dependent and occurred at all doses greater than 100 µg/mL, whereas reduction of MCL-1 in U266 cells was less pronounced, requiring up to 800 µg/mL GCS-100 (Figure 3B).

Apoptosis induction is associated with induction of NOXA expression

Our study suggests crosstalk between the extrinsic and intrinsic apoptosis pathways, an event often mediated by BH3-only proteins. To address this, expression of multidomain and BH3-only proteins with MCL-1/BCL-XL specificity were examined by Western blot analysis. The multidomain proteins BAX and BAK were...
both expressed in nonexposed cells, and levels remained unchanged after exposure to GCS-100 (Figure 3C). The BH3-only proteins BAD, BID, and PUMA were also unchanged in RPMI 8226 cells after incubation with GCS-100 (500 μg/mL). There was, however, a marked increase in expression of NOXA and a small increase in the long and short forms of BIM (BIM/L and BIM/S; Figure 3C).

Caspase inhibition prevents MCL-1 but not BCL-X<sub>L</sub> reduction

To further characterize the reduction in MCL-1 and BCL-X<sub>L</sub>, RPMI 8226 cells were incubated with or without the pan-caspase inhibitor Z-VAD-fmk (50μM) for 1 hour before exposure to GCS-100 (500 μg/mL) for 24 hours (Figure 3D). As predicted, cleavage of caspase-3 and caspase-8 was detected after exposure to
GCS-100 accompanied by reduction in levels of MCL-1 and BCL-XL and increased NOXA levels. No caspase cleavage was detected in cells incubated with Z-VAD-fmk before GCS-100 treatment, confirming inhibition of caspase activation (Figure 3D). In these cells, MCL-1 was not reduced after GCS-100 exposure. However, BCL-XL levels were reduced and NOXA levels increased despite caspase inhibition. This suggests that the GCS-100-induced reduction of MCL-1 is a caspase-dependent event, whereas BCL-XL and NOXA changes are independent of caspase activation.

The uptake of DiOC6(3) and DAPI was also examined in caspase-inhibited cells after 48 hours (Figure 3E-G). Examination of $\Delta A_{DP}$ in viable cells (DAPI-negative) revealed a significant rise in the proportion of DiOC6(3) low cells from 12% to 30% ($P < .001$) when treated with GCS-100 alone with no difference between control cells and cells preincubated with Z-VAD-fmk, suggesting direct caspase-9 involvement. MCL-1 levels remained stable (Figure 3F). In contrast, examination of DAPI-positive or DiOC6(3) low cells, regardless of DAPI, demonstrated a significant increase in necrotic/nonviable cells despite inhibition of caspase activity (Figure 3E,G). The proportion of DAPI-positive cells increased from 19% to 38% when exposed to GCS-100 alone ($P = .002$) and 28% when preincubated with Z-VAD-fmk before GCS-100 exposure ($P = .04$; Figure 3G). The difference between caspase inhibitor-exposed and noncaspase inhibitor-exposed cells treated with GCS-100 was not significant ($P = .1$). The proportion of DiOC6(3) low cells increased from 24% to 52% ($P = .001$). Preincubation with Z-VAD-fmk was associated with a lower proportion of DiOC6(3) low cells (33%), but this was still significantly greater than control cells ($P = .02$; Figure 3E). The difference between GCS-100-treated and Z-VAD-fmk/GCS-100-treated cells was also significant ($P = .01$). Despite caspase inhibition, cell death continues to occur at a reduced level compared with non–caspase-inhibited GCS-100–treated cells. This implies that both caspase-dependent and caspase-independent mechanisms for cell death are involved.

GCS-100 modulates cell-cycle protein expression

We have shown that GCS-100 leads to accumulation of cells in sub-G1 with a significant increase in cells in G1 and an associated fall in cells in S phase and G2/M, suggesting effects on cell-cycle regulation. Expression of proteins associated with regulation of G1 progression, their associated cyclin-dependent and CDK inhibitors were examined. Treatment with GCS-100 led to a down-regulation of Cyclin D2 after 24 hours with an associated reduction in CDK6 and modest reduction in p16INK4A (Figure 4A). CDK4 and p15INK4B were unaffected (Figure 4A). Similarly, a reduction in Cyclin E2 was observed after 4 hours of exposure to GCS-100 together with a moderate reduction in CDK2 and increased expression of p21CIP1 but no change in p27KIP1 (Figure 4B).

Downstream effects on signal transduction

Stimulation of myeloma cells with TNF-α or IL-6 is associated with activation of IKK, IκBα, and NF-κB suggesting induction of signal transduction.18,37 Galectin-3 is also activated by NF-κB, and its expression is associated with NF-κB activation.20 After exposure to GCS-100 for 48 hours, there was a modest reduction of phosphorylated-IκB kinase, whereas nonactivated IKK levels...
Figure 3. GCS-100 is associated with a time- and dose-dependent reduction of critical prosurvival proteins and up-regulation of the proapoptotic protein NOXA. (A) Whole-cell lysates were prepared from RPMI 8226 cells cultured with 500 µg/mL GCS-100 for up to 48 hours, and protein expression was examined by Western blot. A total of 25 µg of protein was separated by 12% SDS-PAGE. Protein was transferred to PVDF membrane and probed with the indicated antibodies to prosurvival BCL-2 family proteins. β-Actin was used to ensure equal loading. (B) RPMI 8226 or U266 cells were exposed to the indicated concentration of GCS-100 for 24 hours. Whole-cell lysates were prepared, and 50 µg of protein was separated by 12% SDS-PAGE, transferred to PVDF membrane, and probed with anti–MCL-1 antibody. β-Actin was used to demonstrate equal protein loading. (C) Whole-cell lysates were prepared from RPMI 8226 cells cultured with 500 µg/mL GCS-100 for up to 48 hours, and protein expression was examined by Western blot. A total of 25 µg of protein was separated by 12% or 15% SDS-PAGE. Protein was transferred to PVDF membrane and probed with the indicated antibodies. β-Actin was used to ensure equal loading. (D) RPMI 8226 cells were incubated with the pan-caspase inhibitor Z-VAD-fmk (50 µM) or dimethyl sulfoxide (DMSO) for 1 hour and then were cultured in the presence or absence of GCS-100 (500 µg/mL) for 24 hours. Protein expression was examined by Western blot (25 µg of protein, 12% SDS-PAGE). The effect of caspase inhibition on GCS-100 mediated loss of mitochondrial transmembrane potential. RPMI 8226 cells were incubated for 1 hour with Z-VAD-fmk or DMSO and then cultured with GCS-100 (500 µg/mL) for 48 hours after which uptake of DiOC6(3) was assessed by flow cytometry as previously described. The proportion of DiOC6(3) low cells (E), DAPI-negative/DiOC6(3) low cells (F), and DAPI-positive cells (G) was assessed. GCS-100 on the x-axis indicates preincubation with DMSO (Z-VAD-fmk control), and Z-VAD-fmk on x-axis indicates preincubation with Z-VAD-fmk followed by GCS-100. Results are mean ± SEM of 3 independent experiments. *Significant difference (P ≤ .05) from untreated control. DiOC6(3).
To determine the impact of GCS-100 on important signaling pathways in myeloma, cell lines were pretreated with GCS-100 before stimulation with the cytokines TNF-α, IGF-1, and IL-6. GCS-100 overcomes the protective effect of IGF-1 and TNF-α

TNF-α activates IκBα with associated reduction of IκBα and activation of NF-κB.37 RPMI 8226 cells were preincubated with GCS-100 for 2 hours with stimulation with TNF-α (5 ng/mL) for 30 minutes. Exposure to TNF-α alone led to an increase in p-IκBα with no changes in p-p65NF-κB, IκBα, or IKK levels (Figure 5C). Cells preincubated with GCS-100 (500 μg/mL) did not increase in p-IκBα after TNF-α stimulation. However, the combination of GCS-100 treatment and TNF-α stimulation was associated with a reduction in IKK and IκBα. No change in p-p65NF-κB levels was observed (Figure 5C).

Myeloma cell stimulation by IGF-1 (100 ng/mL) is associated with activation of AKT.13 However, cells incubated with GCS-100 (500 μg/mL) before IGF-1 stimulation already had markedly reduced levels of p-AKT with no change in AKT levels (Figure 5B).

Figure 4. GCS-100 modulates cell-cycle regulatory proteins. (A-B) Western blot analysis of RPMI 8226 cells cultured with 500 μg of GCS-100 for up to 48 hours. Whole-cell lysates were prepared and 25 μg of protein was separated using 12% or 15% SDS-PAGE and after transfer to PVDF membrane was probed with the indicated antibodies. β-Actin was used to ensure equal protein loading.

Figure 5. GCS-100 treatment is associated with modulation of cell-signaling proteins and inhibits IGF-1 and TNF-α pathway stimulation. (A-B) RPMI 8226 cells were exposed to 500 μg/mL GCS-100 for up to 48 hours and after preparation of whole-cell lysates examined by Western blot. A total of 50 μg of protein was separated using 12% SDS-PAGE, transferred to PVDF membrane, and probed using the indicated antibodies. (C-E) RPMI 8226 cells were incubated in serum-free media for 1 hour and then exposed to 500 μg/mL GCS-100 for up to 48 hours and after preparation of whole-cell lysates examined by Western blot. A total of 50 μg of protein resolved using 12% gel, transferred to PVDF, and probed with the indicated antibodies. β-Actin was used as a loading control.
GCS-100 is a first in class galectin-3 inhibitor. It is shown to induce MCL-1/BCL-X\textsubscript{L} down-regulation with associated NOXA and p21\textsuperscript{Cip1} up-regulation, resulting in concomitant Cyclin E and Cyclin D\textsubscript{2} down-regulation in myeloma cells. In addition, activated NF-\kappaB/AKT is reduced. Overall, this multifactorial response to GCS-100 contributes in a focused manner to positive signals for cell death. Apoptosis is activated via both the extrinsic (caspase-8) and intrinsic (caspase-9) pathways. It may be postulated that carbohydrate binding domains play an important role in myelomagenesis mediated by these antiapoptotic molecules. Targeting these domains with GCS-100 would appear to be an appropriate modality for myeloma therapy, and the good safety profile in a recently reported early clinical trial in chronic lymphocytic leukemia supports its therapeutic potential.

Extending an earlier study,\textsuperscript{27} this work shows activation of caspase-9 as well as caspase-8, with GCS-100 significantly inducing apoptosis and reducing proliferation concomitant with caspase-9 activation. The bimodal caspase activation could suggest a role for the recently discovered P62 protein. Interestingly, prosurvival BCL-2 family proteins MCL-1 and BCL-X\textsubscript{L} are the only down-regulated members of the antiapoptosis proteins. A higher concentration of GCS-100 for the resistant U266 cell line was predictably required. MCL-1 is a critical molecule for myeloma cell survival, making GCS-100 a potentially useful therapeutic molecule. The p53-responsive BH3-only protein PUMA and AKT-regulated BAD bind MCL-1 and BCL-X\textsubscript{L} but were unaltered by GCS-100. However, NOXA, a BH3 only protein that exclusively binds MCL-1 and A1, was rapidly and markedly increased after GCS-100 exposure, suggesting that these proteins are intermediaries for GCS-100 activity. This is consistent with reports that the antimyeloma activity of bortezomib is the result of NOXA up-regulation and MCL-1 cleavage/degradation.\textsuperscript{38,39} Crosstalk between the extrinsic and intrinsic pathways has been previously reported in interferon-\alpha-treated myeloma cells mediated by activated BID, a BH3-only proapoptotic BCL-2 protein, observed to initiate cytochrome c release from mitochondria, thereby initiating intrinsic apoptosis.\textsuperscript{40,41} This study did not detect BID activation, and there was no reduction in full-length BID, suggesting an alternate crosstalk route. Similarly, it has been shown that tumor necrosis factor-related apoptosis-inducing ligand-induced extrinsic pathway activation cleaves caspase-8 inducing MCL-1 degradation, allowing BIM to mediate release of apoptogenic proteins from the mitochondria.\textsuperscript{42} Inhibiting caspase activity with the pan-caspase inhibitor Z-VAD-fmk prevented MCL-1 but not BCL-X\textsubscript{L} reduction or NOXA induction; and although cell death was observed, this was significantly reduced compared with noncaspase-inhibited GCS-100–treated cells. These findings suggest that NOXA induction alone is insufficient to mediate MCL-1 reduction, and caspase activation is required. BCL-X\textsubscript{L} is also reduced despite caspase inhibition, suggesting an alternative regulatory mechanism. NF-\kappaB is a recognized regulator of BCL-X\textsubscript{L}, and its activation is also reduced after GCS-100 exposure. However, the importance of BCL-X\textsubscript{L} in GCS-100–mediated apoptosis is uncertain as its reduction alone is insufficient to induce mitochondrial depolarization. These data all point to significant induction of apoptosis via both caspase pathways after treatment with GCS-100, but additional noncaspase cell death may also occur.

Deregulation of cell-cycle proteins, particularly the Cyclin D proteins in myeloma, have been suggested to be important in the
BLOOD, 13 MAY 2010 • VOLUME 115, NUMBER 19

GCS-100 TARGETS NOXA, MCL-1, AND CELL CYCLE

3947

GCS-100, a galectin-3 antagonist, is a multimodal and beneficial agent against myeloma cells with MCL-1 reduction and cell-cycle inhibition as important therapeutic targets. We provide the logic rationale for clinical testing of GCS-100 for myeloma and other malignancies involving both apoptosis and signal transduction deregulation.

Acknowledgments

The authors thank Dr Paul Allen and Dr Rebecca Auer for reviewing the manuscript and for their helpful comments. GCS-100 was provided as a gift by Prospect Therapeutics.

Authorship

Contribution: M.I.S. designed and performed the research, analyzed the data, and wrote the paper; F.E.C. designed the research, analyzed the data, and wrote the paper; L.M. and S.J. performed primary cell experiments; and J.G.G. and S.A.S. aided experimental design.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Fimbarr E. Cotter, Medical Oncology, John Vane Science Centre, Bart’s & The London School of Medicine, Charterhouse Square, London EC1M 6BQ, United Kingdom; e-mail: f.e.cotter@qmul.ac.uk.

References


GCS-100, a novel galectin-3 antagonist, modulates MCL-1, NOXA, and cell cycle to induce myeloma cell death

Matthew J. Streetly, Lenushka Maharaj, Simon Joel, Steve A. Schey, John G. Gribben and Finbarr E. Cotter