

Calpain activation through galectin-3 inhibition sensitizes prostate cancer cells to cisplatin treatment

Y Wang¹, P Nangia-Makker¹, V Balan¹, V Hogan¹ and A Raz*¹

Prostate cancer will develop chemoresistance following a period of chemotherapy. This is due, in part, to the acquisition of antiapoptotic properties by the cancer cells and, therefore, development of novel strategies for treatment is of critical need. Here, we attempt to clarify the role of the antiapoptotic molecule galectin-3 in prostate cancer cells using siRNA and antagonist approaches. The data showed that Gal-3 inhibition by siRNA or its antagonist GCS-100/modified citrus pectin (MCP) increased cisplatin-induced apoptosis of PC3 cells. Recent studies have indicated that cisplatin-induced apoptosis may be mediated by calpain, a calcium-dependent protease, as its activation leads to cleavage of androgen receptor into an androgen-independent isoform in prostate cancer cells. Thus, we examined whether calpain activation is associated with the Gal-3 function of regulating apoptosis. Here, we report that Gal-3 inhibition by siRNA or GCS-100/MCP enhances calpain activation, whereas Gal-3 overexpression inhibits it. Inhibition of calpain using its inhibitor and/or siRNA attenuated the proapoptotic effect of Gal-3 inhibition, suggesting that calpain activation may be a novel mechanism for the proapoptotic effect of Gal-3 inhibition. Thus, a paradigm shift for treating prostate cancer is suggested whereby a combination of a non-toxic anti-Gal-3 drug together with a toxic chemotherapeutic agent could serve as a novel therapeutic modality for chemoresistant prostate cancers.

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Subject Category: Cancer

Prostate cancer is the most common cancer among men in Western countries. A large number of patients already show metastatic disease at the time of diagnosis. At this stage, prostate cancer is incurable and its growth can only be slowed by hormone deprivation or chemotherapy. Unfortunately, most cases eventually become hormone refractory and chemoresistant.¹ Therefore, it is important to understand the mechanisms for chemoresistance of prostate cancer, and discover new targets to retrigger the sensitivity of prostate cancer cells to chemotherapeutic drugs.

Galectin-3 (Gal-3), a 31 kD carbohydrate-binding protein, is involved in cell growth, cell adhesion, invasion, apoptosis and cancer cell metastasis.^{2–4} Increased expression of Gal-3 has been reported during the progression of several human tumors.^{5–7} In prostate cancer, Gal-3 protein expression may be decreased compared with normal prostate and prostatic intraepithelial neoplasia.^{8,9} Using differential immunohistochemistry, we recently reported that although the expression level of intact Gal-3 decreased, its cleavage could be associated with the progression of prostate cancer.¹⁰ Others have found that loss of Gal-3 expression in early stages of prostate cancer tissues and low aggressive prostate cancer cell lines was due to the heavy methylation of Gal-3 promoter.^{11,12} In addition, Gal-3 knockdown resulted in reduced cell invasion, cell proliferation, and tumorigenicity of PC3 cells.¹⁰ Resistance to apoptosis is one of the hallmarks of cancer cells. The antiapoptotic function of Gal-3 has been well

demonstrated in a variety of human cancers, such as breast,¹³ ovarian,¹⁴ and bladder.¹⁵ However, data regarding the mode of action of Gal-3 in resistance to drug-induced apoptosis of human prostate cancer cells are not yet determined, as only a single study reported that overexpression of Gal-3 in Gal-3 null LNCaP cells resulted in the acquisition of resistance to anticancer drug-induced apoptosis.¹⁶ Another human prostate cancer cell line PC3, which is not sensitive to anticancer drugs, has high expression level of endogenous Gal-3. Therefore, we hypothesized that Gal-3 expression may contribute, in part, to the chemoresistance of prostate cancer, and that inhibition of Gal-3 using siRNA and/or its antagonist may salvage the sensitivity of prostate cancer cells to chemotherapy. GCS-100 AKA, modified citrus pectin (MCP), which is the pH-/temperature-modified form of citrus pectin (CP), is a highly complex branched polysaccharide rich in galactoside residues. CP is water insoluble and is unable to interact with Gal-3, but MCP is water soluble and acts as a ligand for Gal-3 competing with its association to natural ligands.^{17–19} It was reported that GCS-100/MCP could inhibit the lung colonization,¹⁷ cell–cell and cell–matrix interaction of B16-F1 melanoma cells.¹⁸ Oral intake of MCP could inhibit the lung metastasis of prostate cancer cells in rat.¹⁹ GCS-100/MCP feeding inhibited the tumor growth, angiogenesis, and spontaneous metastasis of human breast and colon carcinoma cells in nude mice.²⁰ Jackson *et al* reported that MCP induced apoptosis in human prostate cancer cells.²¹ It was

¹Department of Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, USA

*Corresponding author: A Raz, Department of Pathology, Tumor Progression and Metastasis, Karmanos Cancer Institute, School of Medicine, Wayne State University, 110 East Warren Ave, Detroit, MI 48201, USA. Tel: 313-578-4330; Fax: 313-831-7518; E-mail: raza@karmanos.org

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Abbreviations: Gal-3, galectin-3; MCP, modified citrus pectin; CP, citrus pectin; PARP, poly (ADP-ribose) polymerase; SDS, sodium dodecyl sulfate; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin

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also shown that GCS-100/MCP either alone or in combination with dexamethasone inhibits myeloma cells growth, and overcomes drug resistance.²²

Cisplatin, a widely used chemotherapeutic agent, is highly effective against several cancers, including testicular, breast, ovarian, bladder, and lung cancers. Late stage prostate cancer is resistant to cisplatin treatment because of the development of chemoresistance.²³ Cisplatin treatment in Gal-3-expressing PC3 cells could serve as a model to study the relationship between Gal-3 expression and chemoresistance of prostate cancer cells. Two major apoptotic pathways have been defined: death receptor apoptotic pathway leading to caspase-8 activation and mitochondrial apoptotic pathway leading to cytochrome c release and caspase-9 activation.²⁴ Both pathways activate downstream effector caspases (caspase-3, -6, and -7) that lead to apoptosis features. Of note, in prostate cancer, it was reported that apoptosis can be mediated by calpain activation.^{25,26} Calpain is a family of calcium-dependent proteases, calpain 1 and 2 are major family members. Studies suggest that apoptosis may be mediated by calpain activation in response to the alteration of mitochondria-mediated calcium homeostasis.²⁷ Calpain activation may lead to the cleavage of androgen receptor into an androgen-independent isoform in apoptotic prostate cancer cells.²⁸ Here, we report that inhibition of Gal-3 using siRNA or its antagonist GCS-100/MCP increased cisplatin-induced apoptosis of PC3 cells, and calpain activation contributed to the proapoptotic effect of Gal-3 inhibition.

Results

Gal-3 knockdown sensitized PC3 cells to chemotherapeutic drug-induced apoptosis. RNAi technique was used to knockdown Gal-3 expression, and the expression levels of Gal-3 protein in two transfectants were sharply reduced (Figure 1a). Furthermore, the lower panel shows that Gal-3 protein mainly localized in the cytoplasm of PC3 cells. Figure 1b shows that Gal-3 knockdown boosts apoptosis of PC3 cells treated by 50 μ M of cisplatin or 6 nM of docetaxel. The proapoptotic effect of Gal-3 knockdown was further confirmed by detection of 89 kD poly (ADP-ribose) polymerase (PARP), DNA laddering assay, cell viability assay, and DNA content assay by flow cytometry. PARP, a 113 kD protein, is cleaved to fragments of \sim 89 and 24 kD during apoptosis,²⁹ and detection of 89 kD PARP has served as a marker of apoptosis.³⁰ As shown in Figure 1c, compared with VC cells, significantly increased levels of 89 kD PARP were detected in siGal3-11 and siGal3-19 after cisplatin treatment. Figure 1d shows more DNA fragmentation was

detected in Gal-3 knockdown cells after cisplatin treatment. Cell viability assay also showed that fewer cells survived in siGal3-11 (40% \pm 3.6) and siGal3-19 (26.6% \pm 4.5) clones compared with VC cells (53.8% \pm 5.8) after cisplatin treatment (Figure 1e). In Figure 1f, cisplatin treatment resulted in more sub-G1 region cells, indicating more apoptotic cells in Gal-3 knockdown clones.

Gal-3 knockdown potentiated cisplatin-induced caspase activation. Western blot analysis of the expression of active caspase-3 (Figure 2ai) and caspase-3 activity assay (Figure 2aii) suggested that Gal-3 knockdown transfectants showed a higher level of caspase-3 activation than control cells after cisplatin treatment. As shown in Figure 2b, cisplatin treatment resulted in caspase-9 activation in Gal-3 knockdown transfectants, but no caspase-9 activation was observed in control cells. The expression of Gal-3 protein is not changed by cisplatin treatment (Figure 2c).

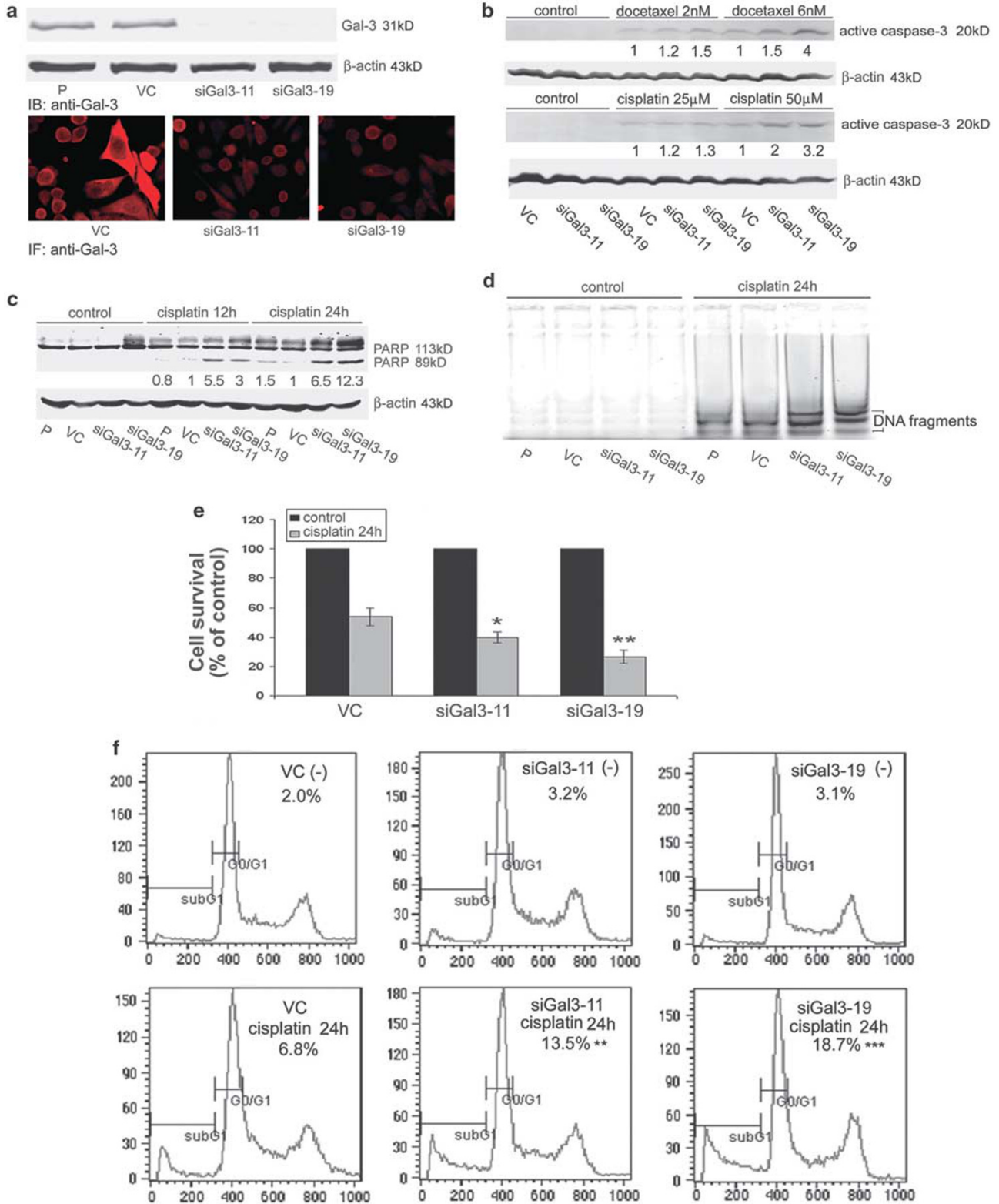
Gal-3 knockdown facilitated mitochondrial apoptotic events. Mitochondria were stained with mitochondrion-selective probe MitoTracker Red CMXRos. As observed in untreated cells (Figures 3A, a, b, and c), the mitochondria in cisplatin-treated VC cells (Figures 3A, d) retained the fibrillar fluorescence pattern and strong staining. However, 6 h of cisplatin treatment resulted in the loss of mitochondrial structure and reduced staining in siGal3-11 and siGal3-19 cells (Figures 3A, e and f), as MitoTracker Red dye is washed out of cells and cannot be sequestered once mitochondrial membrane potential is lost. Our results also show that levels of cytosolic cytochrome c in Gal-3 knockdown cells were elevated up to 3.8-fold compared with that in VC cells after cisplatin treatment (Figure 3B). To explore what contributes to Gal-3-associated mitochondrial protection, western blot analysis of Bcl-2 family members was performed and results show that cisplatin treatment increased Bcl-2 expression in Gal-3-expressing VC cells; however, in Gal-3 knockdown cells, Bcl-2 expression was not affected by cisplatin treatment and still remained at the basic level as untreated cells. The expression level of Bcl-X_L or Bax between control cells and Gal-3 knockdown transfectants did not show obvious differences (Figure 3C).

Loss of Gal-3 expression enhanced cisplatin-induced calpain activation. Recent studies have demonstrated that activation of calpain could mediate apoptosis.^{25,26} We examined calpain activation by western blot analysis of its substrate spectrin α II and zymography analysis of its substrate casein. Spectrin α II is an universally expressed membrane-associated cytoskeletal protein.³¹ Full-length

Figure 1 Gal-3 knockdown enhanced chemotherapeutic drug-induced apoptosis of PC3 cells. (a) Gal-3 expression and localization were analyzed by western blot analysis (upper) and immunofluorescence (400 \times magnification) (lower). (b) The effect of Gal-3 knockdown on caspase-3 activation induced by different concentrations of drugs. Cells were treated with docetaxel (2 nM or 6 nM) and cisplatin (25 μ M or 50 μ M) for 48 h. Numbers represent the relative intensity of active caspase-3 normalized to β -actin. The values of VC cells were set as "1". (c) The effect of Gal-3 knockdown on PARP cleavage induced by cisplatin (50 μ M) for indicated times. Numbers represent the relative intensity of 89 kD PARP normalized to β -actin. The values of VC cells were set as "1". DNA laddering assay (d), cell viability assay by MTT (e), and DNA content assay by flow cytometry (f) confirmed the proapoptotic effect of Gal-3 knockdown. Cells were treated with cisplatin (50 μ M) for 24 h. In (e), columns ($n=3$), mean; bars, \pm S.D. * $P<0.05$; ** $P<0.01$ compared with VC cells. In (f), the percentage represents apoptotic cells in sub-G1 region. ** $P<0.01$; *** $P<0.001$ compared with VC cells. P, parental cells; VC, cells transfected with non-target vector; siGal3-11 and siGal3-19, Gal-3 knockdown clones. Data are representative of three independent experiments

spectrin α II can be cleaved into a 150kD fragment specifically by calpain³² or a 120kD fragment specifically by caspase-3.³³ Figure 4A showed that cisplatin treatment did not change the expression level of either calpain 1 or 2.

In Figure 4B, cisplatin treatment for 12 h resulted in elevated levels of 150 kD fragments of spectrin in siGal3-11 (3.4-fold) and siGal3-19 (3.2-fold). Interestingly, 24 h after cisplatin treatment, 150 kD fragments in Gal-3 knockdown cells



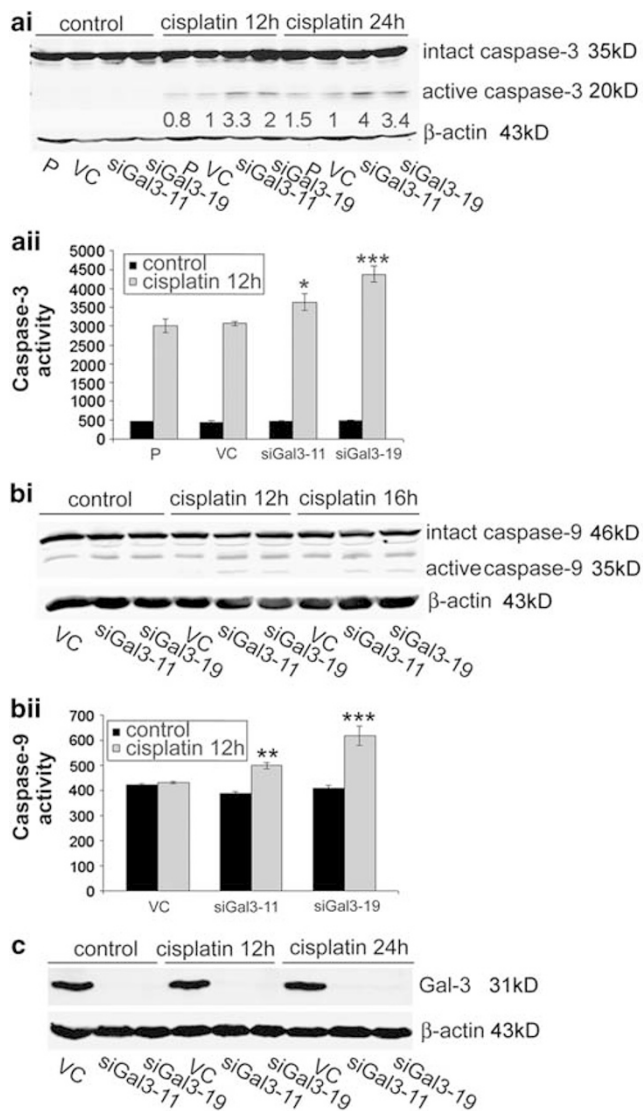


Figure 2 Gal-3 knockdown enhanced cisplatin-induced activation of caspase-3 and -9. Cells were treated with $50 \mu\text{M}$ cisplatin for indicated times. Western blot analysis of the expression levels of active caspase-3 (ai) and caspase-9 (bi). Numbers represent the relative intensity of active caspase-3 normalized to β -actin. The values of VC cells were set as '1'. (a(ii)) caspase-3 activity assay and (b(ii)) caspase-9 activity assay. Columns ($n=3$), mean; bars, \pm S.D. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared with VC cells. (c) Western blot analysis of Gal-3 expression. Data are representative of three independent experiments

became slightly lower than that in VC cells, it may be possible that 150 kD fragments can be further cleaved by active caspase-3 into 120 kD fragments. In addition, casein zymography showed that 12h cisplatin treatment resulted in higher levels of calpain 2 activity in siGal3-11 (4.9-fold) and siGal3-19 (5.1-fold) than that in VC cells (Figure 4C). Our data also indicate that Gal-3 knockdown increased cisplatin-induced intracellular calcium levels (Figure 4D). To confirm the regulatory effect of Gal-3 on calpain activation, we also examined the activation of calpain in LNCaP cells. It was reported that Gal-3 overexpression reduced cisplatin-induced apoptosis of LNCaP cells.¹⁶ Here, as shown in

Figure 5c, 150 kD fragment of spectrin indicating calpain activation was detected in Gal-3 null VC cells after cisplatin treatment, but not in Gal-3 overexpressing clones. Cisplatin treatment resulted in higher level of calpain activity in Gal-3 null VC cells compared with Gal-3 overexpressing clones (Figure 5d). The expression levels of calpain 2 in control cells and Gal-3 overexpressing clones were not changed by cisplatin (Figure 5b).

Calpain activation contributed to the proapoptotic effect of Gal-3 knockdown. Two independent methodologies (specific calpain inhibitor PD150606 and siRNA) were employed to inhibit calpain activation. Figure 6ai indicates that cisplatin-induced calpain activation in Gal-3 knockdown cells was attenuated to 10% by $20 \mu\text{M}$ PD150606. This dosage of PD150606 also attenuated cisplatin-induced caspase-3 activation ($<70\%$ in siGal3-11 and $<60\%$ in siGal3-19) and cleavage of PARP ($<70\%$ in siGal3-11 and $<80\%$ in siGal3-19) in Gal-3 knockdown cells, but had no effect on cytochrome c release or caspase-9 activation (Figure 6a(ii)), suggesting that calpain activation might be a novel mechanism for the proapoptotic effect of Gal-3 knockdown through inducing caspase-3 activation in a mitochondria-independent way. Furthermore, we transfected cells with siRNAs targeting calpain 1 or 2 to further understand which isoform exerts the effect. Transfection of calpain 1 siRNA had no effect on apoptosis. Figure 6bi showed the inhibitory efficiency of siRNA targeting calpain 2. Transfection of calpain 2 siRNA attenuated the enhanced caspase-3 activation ($<60\%$) and PARP cleavage ($<70\%$ in siGal3-11 and $<80\%$ in siGal3-19) in Gal-3 knockdown cells (Figure 6b(ii)), suggesting that calpain 2 activation mediated the proapoptotic effect of Gal-3 knockdown in PC3 cells.

Inhibition of Gal-3 by GCS-100/MCP-enhanced cells' sensitivity to cisplatin in a calpain-dependent way. To confirm the association of Gal-3 expression with resistance to cisplatin, GCS-100/MCP was used to block the functions of Gal-3. GCS-100/MCP sensitized Gal-3-expressing PC3 cells to cisplatin-induced cell death (Figure 7ai). Compared with cisplatin only, the expression levels of active caspase-3 elevated up to 4.6-fold in cells treated with cisplatin and GCS-100/MCP together (Figure 7a(ii)). These results indicated that GCS-100/MCP may confer cells' sensitivity to cisplatin-induced apoptosis through blocking Gal-3 functions. Figure 7bi showed that the expression levels of spectrin 150 kD fragments in cells treated with GCS-100/MCP and cisplatin together were up to 3.2-fold higher than cells treated with cisplatin only, this was confirmed with casein zymography (Figure 7b(ii)). In Figure 7c, enhanced cisplatin-induced apoptosis by GCS-100/MCP was attenuated by calpain 2 siRNA, suggesting that the proapoptotic effect of Gal-3 inhibition through GCS-100/MCP was mediated by calpain activation.

Discussion

Prostate cancer is the second leading cause of cancer-related death in Western countries.³⁴ The early stages of prostate cancer can be effectively treated by androgen ablation

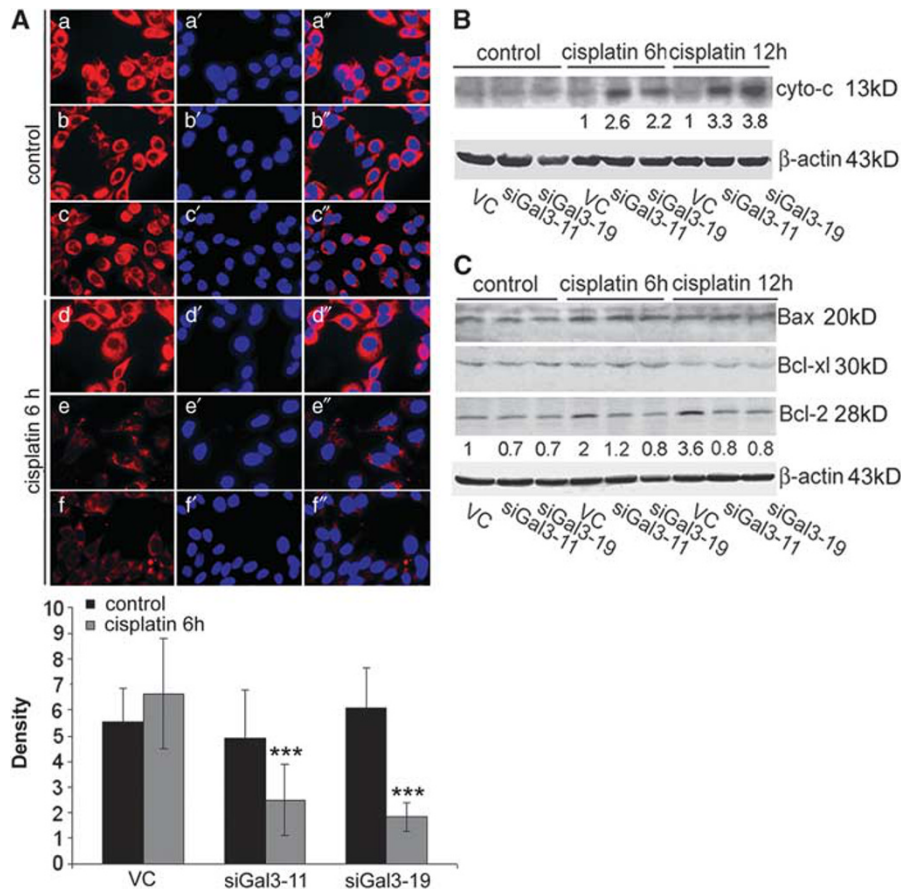


Figure 3 Gal-3 knockdown enhanced cisplatin-induced mitochondrial apoptotic events. Cells were treated with 50 μ M cisplatin for indicated times. (A) Fluorescent image of mitochondria staining (400 \times magnification). Mitochondria were stained as indicated in Materials and Methods. a–f, mitochondria staining with MitoTracker Red CMXRos; a'–f', nuclear staining with 4', 6-diamidino-2-phenylindole; a''–f'', merged image. a–a'' and d–d'', VC; b–b'' and e–e'', siGal3-11; c–c'' and f–f'', siGal3-19. Lower panel is the quantification of fluorescent density of mitochondria staining using Adobe Photoshop CS3 extended software (Adobe Systems Incorporated, San Jose, CA, USA). *** P <0.001, compared with VC cells. Western blot analysis of cytochrome *c* release (B) and Bcl-2 family members (C). Numbers represent the relative intensities of cytochrome *c* and Bcl-2 normalized to β -actin. The values for cytochrome *c* and Bcl-2 of VC cells were set as "1". Data are representative of three independent experiments

therapy. However, most cases of prostate cancer eventually relapse to an androgen refractory state at which chemotherapy is the final choice to slow down the growth of prostate cancer. Unfortunately, chemotherapy is not effective because cancer cells develop resistance to drugs. Therefore, it is imperative to explore novel therapeutic targets to improve the sensitivity of prostate cancer cells to chemotherapy.

Gal-3, a β -galactoside-binding protein, regulates multiple biological functions, including regulating cell proliferation, invasion, tumorigenicity, and apoptosis. It was reported that decreased expression and increased cleavage of Gal-3 are associated with the development and progression of human prostate cancer.^{8–10} Exogenously added recombinant Gal-3 exhibits proapoptotic activity in human T leukemia cells,³⁵ whereas intracellular Gal-3 imparts resistance to apoptosis in many cancer cells. For example, overexpression of Gal-3 protected human breast cancer cell line BT-549 from apoptosis through inducing cell cycle arrest.³⁶ Overexpression of Gal-3 inhibits tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human bladder carcinoma cells.¹⁵ However, the association of Gal-3 with the resistance to drug-induced apoptosis of prostate cancer cells

is not yet well documented. It was reported that Gal-3 overexpression in Gal-3 null LNCaP cells resulted in the acquisition of resistance to anticancer drug-induced apoptosis.¹⁶ On the other hand, PC3 cells express high level of endogenous Gal-3 and are not sensitive to chemotherapeutic agents. The opposite properties between LNCaP and PC3 cells lead us to hypothesize that expression of Gal-3 might partly be responsible for the resistance to drug-induced apoptosis of prostate cancer cells.

To testify our hypothesis, we studied the effect of inhibition of Gal-3 on cisplatin-induced apoptosis using two approaches: Gal-3 knockdown by siRNA and blockage of Gal-3 functions using GCS-100/MCP. GCS-100/MCP was reported to have inhibitory effects on tumor growth, homotypic and heterotypic cell adhesion of cancer cells, tumor metastasis, and induced apoptosis of cancer cells by acting as an antagonist of Gal-3.^{17–21} As reported, GCS-100/MCP was already used in clinical trials for treating various tumors.³⁷ Our results showed that Gal-3-expressing cells were resistant to chemotherapeutic drug-induced apoptosis as compared with Gal-3 knockdown cells, indicating that Gal-3 expression may be associated with chemoresistance of PC3 cells and

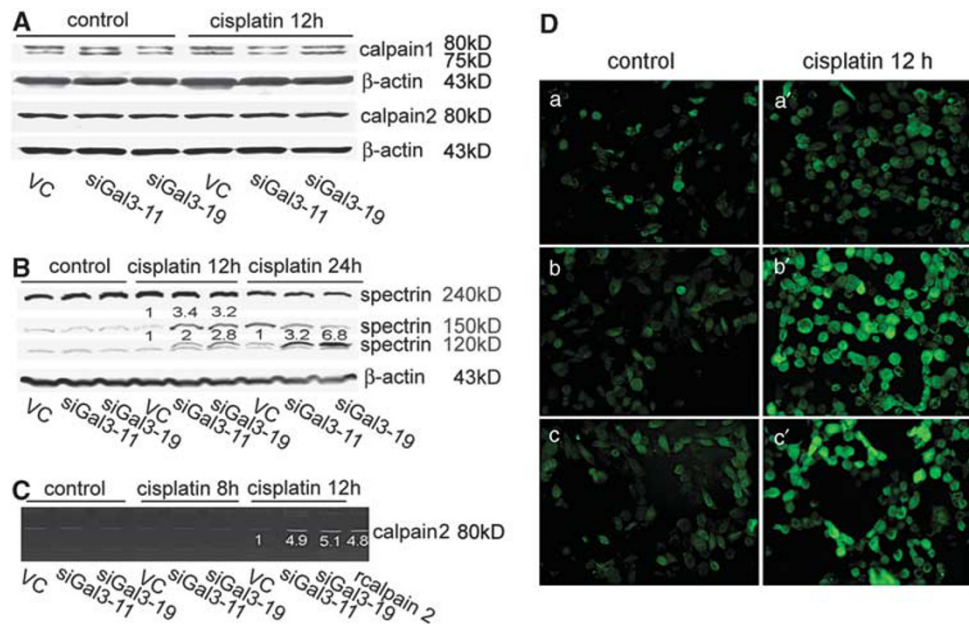


Figure 4 Gal-3 knockdown enhanced cisplatin-induced calpain activation in PC3 cells. Cells were treated with 50 μ M cisplatin for indicated times. (A) Western blot analysis showed that cisplatin did not change the expression levels of calpain 1 and 2. (B) Calpain activity as represented by western blot analysis of spectrin 150 kD fragment. Numbers represent the relative intensities of spectrin 150 kD and spectrin 120 kD normalized to β -actin. The values of VC cells were set as "1". (C) Casein zymography to detect calpain activity. Purified recombinant rat calpain 2 was used as the positive control. Numbers represent the intensity of calpain 2. The value of VC cells was set as "1". (D) Fluorescent image of cytosolic calcium levels (200 \times magnification). Cells were stained with Fluo-4 AM as indicated in Materials and Methods. a and a', VC; b and b', siGal3-11; c and c', siGal3-19. Data are representative of three independent experiments

inhibition of Gal-3 may render the retrieval of chemosensitivity of prostate cancer cells. Furthermore, we determined that the increased apoptosis mediated by Gal-3 knockdown was due to mitochondrial damage, leading to cytochrome c release and activation of caspase-9. This is consistent with the previous study on LNCaP cells.¹⁶ The Bcl-2 family consists of both proapoptotic (Bax and Bak) and antiapoptotic (Bcl-2 and Bcl-X_L) members.³⁸ Here, we report that Gal-3 knockdown alters the expression level of neither Bcl-X_L nor Bax. In Gal-3-expressing VC cells, cisplatin treatment resulted in upregulation of Bcl-2, which is the protective factor for mitochondrial integrity. However, in Gal-3 knockdown cells, the expression levels of Bcl-2 after cisplatin treatment remained at the basic level as observed in untreated cells, suggesting that Gal-3 knockdown prevents Bcl-2 upregulation induced by cisplatin and consequent protection of mitochondrial integrity. To confirm the effect of Gal-3 knockdown on apoptosis, GCS-100/MCP was used to block the function of Gal-3, and our results suggest that GCS-100/MCP may enhance cells' sensitivity to cisplatin through the inhibition of Gal-3.

In addition to the death receptor and mitochondrial apoptotic pathways, increasing evidence indicates the association of calpain activation with apoptosis of prostate cancer cells.^{25,26} Calpain is a family of calcium-dependent proteases that are functionally active as a heterodimer composed of a small regulatory subunit and one large catalytic subunit. Calpain 1 (requires micromolar levels of calcium for activation) and 2 (requires millimolar levels of calcium for activation) are the major members of this family and have been studied most extensively. PC3 cells can express both calpain 1 and 2. It has been reported that calpain activation mediated

cisplatin-induced apoptosis in lung cancer cells,³⁹ and calpain activation could be triggered in response to alteration of mitochondria-mediated calcium homeostasis.²⁷ Studies also showed that cisplatin-induced calpain activation mediated androgen receptor breakdown in apoptotic prostate cancer cells.²⁸ Our results demonstrated that non/low Gal-3-expressing cells exhibited higher level of calpain activity than high Gal-3-expressing cells in response to cisplatin treatment (Figures 4 and 5), suggesting that calcium-dependent calpain activation may be a novel mechanism for the proapoptotic effect of Gal-3 knockdown on cisplatin-induced apoptosis in prostate cancer cells. Using specific inhibitor PD150606 and/or siRNAs targeting calpain 1 or 2, we revealed that cisplatin-induced PARP cleavage and caspase-3 activation in Gal-3 knockdown cells were attenuated by PD150606 or calpain 2 siRNA, neither caspase-9 activation nor cytochrome c release was affected, which indicates that calpain activation (specifically calpain 2 activation) mediated proapoptotic effect of Gal-3 knockdown on cisplatin-induced apoptosis in a mitochondria-independent way in prostate cancer cells. This was further confirmed by using GCS-100/MCP. GCS-100/MCP treatment enhanced cisplatin-induced calpain activation through blocking the functions of Gal-3, and the proapoptotic effect of Gal-3 inhibition by GCS-100/MCP was mediated by calpain 2 activation.

Our study suggested that Gal-3 expression contributes, in part, to the chemoresistance of prostate cancer cells, and Gal-3 inhibition by siRNA or GCS-100/MCP could resensitize prostate cancer cells to cisplatin treatment through mitochondrial apoptotic pathway and calpain activation. To conclude, the data suggest a paradigm shift in treating prostate cancer

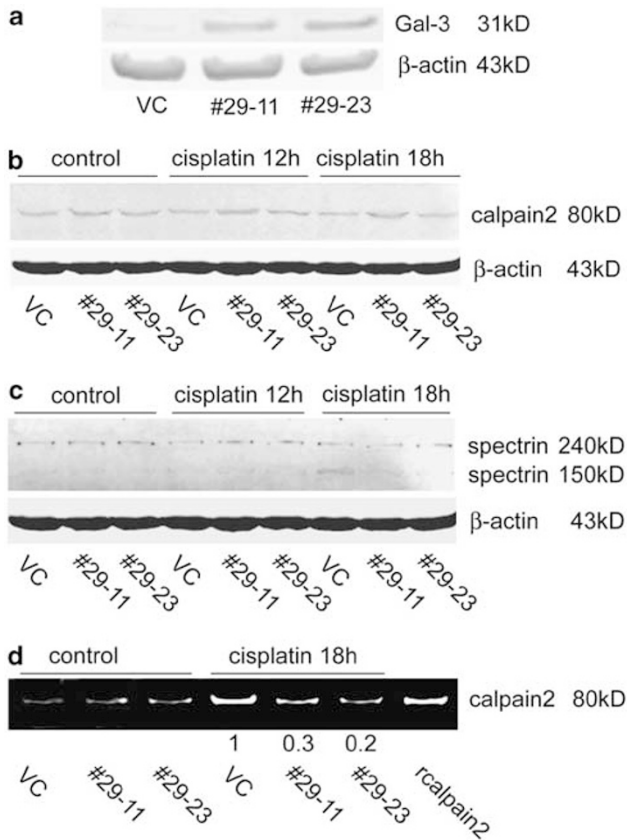


Figure 5 Gal-3 overexpression reduced cisplatin-induced calpain activation in LNCaP cells. Cells were treated with 50 μ M cisplatin for indicated times. (a) The expression levels of Gal-3 in control LNCaP and Gal-3 overexpressing clones. (b) Western blot analysis showed that cisplatin had no effect on the expression level of calpain 2. (c) Calpain activity as represented by western blot analysis of spectrin 150 kD fragment. (d) Casein zymography to detect calpain activity. Purified recombinant rat calpain 2 was used as the positive control. Numbers represent the intensity of calpain 2. The value of VC cells was set as “1”. VC, cells transfected with control vector; #29-11 and #29-23, two Gal-3 overexpressing clones. Data are representative of three independent experiments

whereby a combination therapy of a non-toxic polysaccharide Gal-3 antagonist together with a toxic chemotherapeutic agent could serve as a novel therapeutic modality for chemorefractory prostate cancers.

Materials and Methods

Materials and antibodies. Monoclonal rat anti-Gal-3 antibody was isolated from the supernatant of hybridoma (catalog number: TIB-166, American Type Culture Collection, Rockville, MD, USA); cisplatin, docetaxel, thiazolyl blue tetrazolium bromide (MTT), mouse anti- β -actin and anti- β -tubulin were purchased from Sigma Chemicals (St. Louis, MO, USA); fluorogenic caspase substrates Ac-DEVD-AMC and Ac-LEHD-AFC, calpain-specific inhibitor PD150606, and mouse anti-Bcl-X_L were purchased from Calbiochem (San Diego, CA, USA); MitoTracker red CMXRos and Fluo-4 AM were purchased from Invitrogen (Carlsbad, CA, USA); siRNAs duplex targeting calpain 1, 2 and non-target control siRNA, rabbit anti-caspase-3, rabbit anti-caspase-9, and mouse anti-spectrin α II were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti-PARP (Biomol, Plymouth Meeting, PA, USA); FITC rabbit anti-active caspase-3 (BD Biosciences, San Diego, CA, USA); mouse anti-cytochrome *c* (Zymed Laboratories, South San Francisco, CA, USA); rabbit anti-Bcl-2, anti-Bax and anti-calpain 2 (Cell Signaling Technology, Beverly, MA, USA).

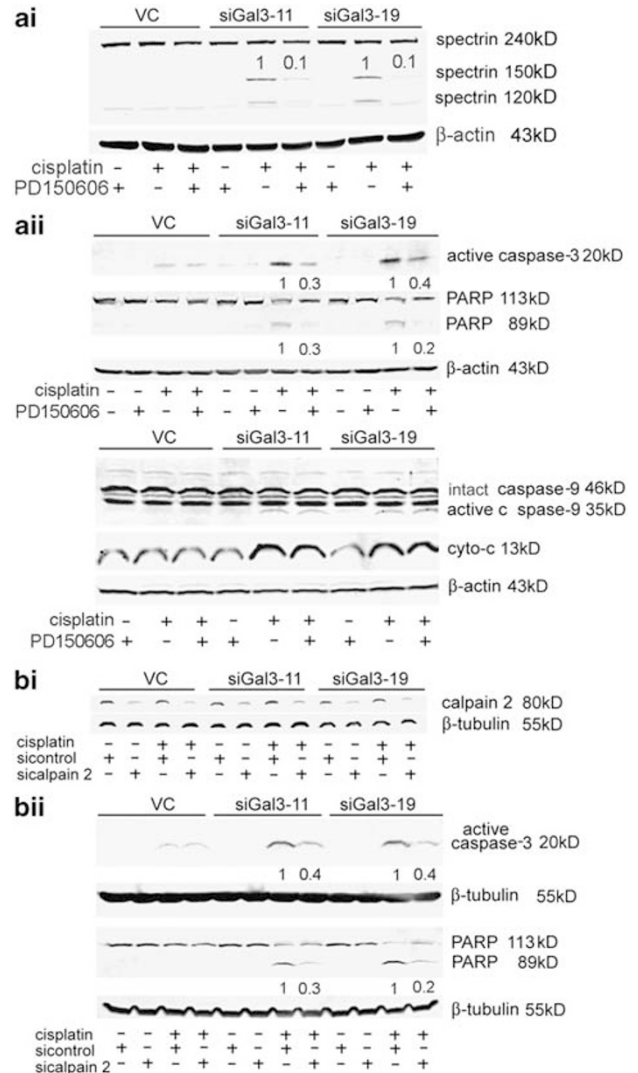


Figure 6 Calpain activation contributed to the proapoptotic effect of Gal-3 knockdown. Cells were pretreated with 20 μ M PD150606 for 30 min and then treated with 50 μ M cisplatin for 12 h. (ai) Inhibitory effect of PD150606 on calpain activity. Numbers represent the relative intensity of spectrin 150 kD normalized to β -actin. The values of siGal3-11 and siGal3-19 treated with cisplatin only were set as “1”. (aII) The effect of PD150606 on caspase-3 activation, PARP cleavage, caspase-9 activation, and cytochrome *c* release. Numbers represent the relative intensities of active caspase-3 and PARP 89 kD normalized to β -actin. The values of siGal3-11 and siGal3-19 treated with cisplatin only were set as “1”. (bi) Downregulation of calpain 2 expression by siRNA transfection. After 24 h of transfection, cells were treated with 50 μ M cisplatin for 12 h. (bii) Calpain 2 knockdown attenuated cisplatin-induced caspase-3 activation and PARP cleavage. Cells were treated as the same way as bi. Numbers represent the relative intensities of active caspase-3 and PARP 89 kD normalized to β -tubulin. The values of siGal3-11 and siGal3-19 with sicontrol transfection and cisplatin treatment were set as “1”. Data are representative of three independent experiments

Preparation of GCS-100/MCP. CP was purchased from Sigma Chemicals. Temperature modification of CP was performed as follows: CP solution (1.3%) was autoclaved for 1 h, cooled to room temperature, centrifuged at 10 000 r.p.m. for 10 min. Collected supernatant was precipitated with two volumes of absolute ethanol and frozen at -20° C for a minimum of 2 h. After centrifuging at 10 000 r.p.m. for 10 min again, the supernatant was discarded and pellet was saved. The pellet was resuspended in acetone, filtered, and dried on Whatman filters. MCP

was dissolved in de-ionized distilled water and 0.3% MCP was used as the working concentration.

Cell culture. Human prostate cancer cells PC3 (ATCC CRL-1435) and LNCaP (ATCC CRL-1740) were purchased from the American Type Culture Collection (Manassas, VA, USA). Two Gal-3 knockdown transfectants of PC3 cells named as siGal3-11 and siGal3-19, and one non-target control vector transfectant named as VC were established as described in our previous study.¹⁰ Two Gal-3 over-expressing clones of LNCaP cells named as #29-11 and #29-23, and one non-target control vector clone named as VC were kindly provided by Dr. Reuben Lotan

(University of Texas MD Anderson cancer center, Houston, Texas, USA). PC3 and LNCaP cells were cultured in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, supplemented with 10% fetal bovine serum. For transfectants, 200 $\mu\text{g}/\text{ml}$ G418 (Invitrogen) was added to the culture medium.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde/PBS for 15 min, permeabilized with 0.2% Triton X-100/PBS for 10 min, blocked in 1% bovine serum albumin (BSA)/PBS for 30 min and incubated with rat anti-Gal-3 antibody (1:50) for 1 h, then incubated with Texas red-conjugated anti-rat antibody (1:2000, ICN Biomedicals, Costa Mesa, CA, USA) for 1 h in the dark. Cells were mounted in gelvatol and photographed at the same parameter using Sony DXC-979MD 3CCCD video camera (Tokyo, Japan) connected to an OLYMPUS BX40 microscope (Melville, NY, USA).

Assessment of apoptosis

DNA laddering assay. Cell pellets were lysed with TE/Triton buffer (10 mM Tris (pH 8.0), 0.2% Triton X-100, 1 mM EDTA) on ice for 10 min, centrifuged at $13000 \times g$ for 15 min at 4°C . This lysis buffer is so mild that it cannot lyse nuclei, so the supernatant only contains the low-molecular weight DNA fragments released from nuclei; therefore, no high-molecular weight DNA is observed. The same amount of supernatant was incubated at 37°C for 1 h with addition of 70 $\mu\text{g}/\text{ml}$ RNase A, incubated at 50°C for 2 h with addition of 0.5% sodium dodecyl sulfate (SDS) and 500 $\mu\text{g}/\text{ml}$ proteinase K, precipitated with ice-cold isopropanol, centrifuged at $13000 \times g$ for 15 min at 4°C . DNA pellets were dissolved in TE buffer. DNA solutions were run on a 2% agarose gel containing 100 nM SYTO 60 red fluorescent nucleic acid stain (Invitrogen). The gel was scanned using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Cell viability assay. Cell viability was assessed by MTT as described previously.¹⁵ Cell survival of treated cells was represented as the percentage of untreated control cells.

Detection of 89 kD PARP and active caspase-3. Cells pellets were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl) containing protease inhibitors. The resulting cell lysate was analyzed by western blot using anti-PARP antibody and anti-caspase-3 antibody.

DNA content assay by flow cytometry. Briefly, a total of 1×10^6 cells were collected. After washing, cells were fixed with 80% ethanol overnight at -20°C , washed with PBS, and treated with RNase A (1 mg/ml) for 15 min at 37°C followed by staining with propidium iodide (50 $\mu\text{g}/\text{ml}$) (Sigma) for 15 min at room temperature. DNA content was analyzed using a Becton Dickinson FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Caspase activity assay. Caspase activity was measured using fluorogenic caspase substrates as described previously.¹⁶ Cell pellets were lysed with cell

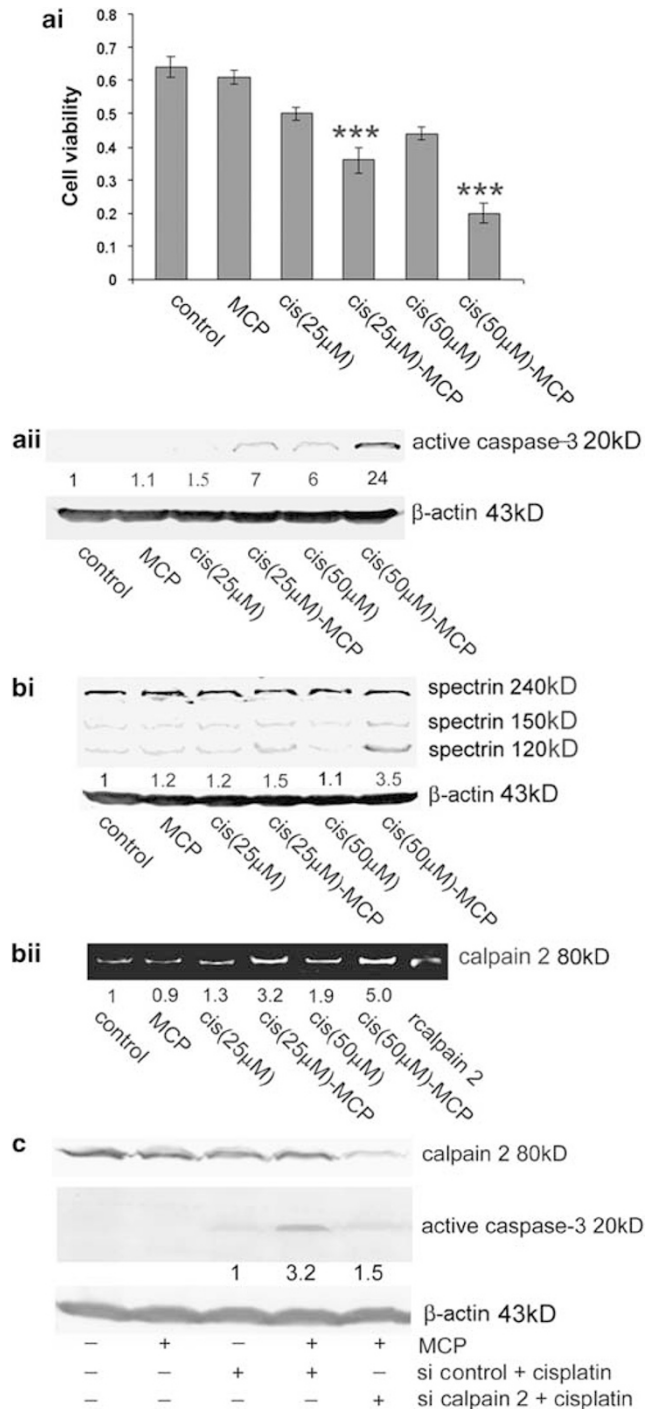


Figure 7 The blockage of Gal-3 by GCS-100/MCP enhanced cisplatin-induced apoptosis in a calpain-dependent way. **(ai)** Cell viability assay by using MTT. Columns (n=3), mean; bars, \pm S.D. *** $P < 0.001$ compared with cisplatin treatment only. **(aia)** Western blot analysis of active caspase-3. Numbers represent the relative intensity of active caspase-3 normalized to β -actin. The value of control cells was set as "1". Parental PC3 cells were pretreated with 0.3% MCP for 30 min and then cisplatin for 24 h. **(bi)** Calpain activity as represented by western blot analysis of spectrin 150 kD. Numbers represent the relative intensity of spectrin 150 kD normalized to β -actin. The value of control cells was set as "1". **(bii)** Casein zymography to detect calpain activity. Purified recombinant rat calpain 2 was used as the positive control. Numbers represent the intensity of calpain 2. The value of control cells was set as "1". Parental PC3 cells were pretreated with 0.3% MCP for 30 min and then cisplatin for 12 h. **(c)** Western blot analysis of active caspase-3 showed that calpain 2 knockdown attenuated the proapoptotic effect of Gal-3 blockage by GCS-100/MCP. Parental PC3 cells were transfected with siRNA duplex targeting calpain 2 or non-target control siRNA. After 24 h of transfection, cells were pretreated with 0.3% MCP for 30 min, and then treated with cisplatin (50 μM) for 24 h. Numbers represent the relative intensity of active caspase-3 normalized to β -actin. The value of cells transfected with control siRNA plus cisplatin treatment was set as "1". Data are representative of three independent experiments

extract buffer (20 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.03% nonidet P-40) on ice for 30 min. Lysates were centrifuged at 15 000 × *g* for 10 min at 4°C, and 25 μg of cell lysates were incubated at 37°C for 1 h in a total volume of 100 μl of caspase buffer (10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT) containing 35 μM Ac-DEVD-AMC or Ac-LEHD-AFC. Fluorescence upon cleavage of AMC or AFC by activated caspases was monitored (excitation: 380 nm for AMC and 400 nm for AFC, emission: 460 nm for AMC and 505 nm for AFC) using a Spectra Maxi Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

Mitochondrial staining. Mitochondrial integrity was assessed by labeling mitochondria using a mitochondrion-selective probe according to the manufacturer's instruction. Briefly, cells were incubated at 37°C for 30 min in growth medium containing 100 nM MitoTracker red CMXRos, fixed with 3.7% formaldehyde in growth medium at 37°C for 15 min. Then, cells were stained with 4', 6-diamidino-2-phenylindole solution for 1 min. Slides were mounted in gelvatol and photographed at the same parameter using Sony DXC-979MD 3CCCD video camera connected to an OLYMPUS BX40 microscope.

Cytochrome c release. Cells were lysed with mild cell extraction buffer (20 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 4 mM DTT, and 250 mM sucrose) on ice for 1 h. The lysates were then passed through a 27^{1/2}-gauge syringe 10 times and centrifuged at 15 000 × *g* for 15 min at 4°C as described previously.¹⁶ The resulting supernatant was analyzed by western blot using mouse anti-cytochrome c antibody.

Casein zymography. Calpain activity was measured by zymography using its enzyme substrate casein as described by Raser *et al.*⁴⁰ Briefly, cell pellets were lysed with mild cell extract buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, and 250 mM sucrose) containing protease inhibitors on ice for 30 min, then passed through a 27^{1/2}-gauge syringe 10 times, and incubated on ice for another 30 min. Pure cytosolic fractions were cleared from cell lysate by ultracentrifugation at 100 000 × *g* for 1 h at 4°C. To cast casein zymography gels, casein (0.2% w/v) was copolymerized with 12% acrylamide, 0.32% bis-acrylamide, 375 mM Tris-HCl (pH 8.8) as the separating gel and 4% acrylamide, 0.11% bis-acrylamide, and 330 mM Tris-HCl (pH 6.8) (no casein) as the stacking gel. The gel was pre-run at 130 V for 30 min at 4°C in running buffer (25 mM Tris base and 192 mM glycine), then 60 μg of each sample mixed with 1/5 volume of sample buffer (150 mM Tris-HCl (pH 6.8), 20% glycerol, and 0.004% bromophenol blue) was loaded to the gel, and run at 80 V for 2 h at 4°C. After running, the gel was rinsed twice with incubation buffer (20 mM Tris-HCl, 10 mM DTT, and 3 mM CaCl₂ (pH 7.5)) and incubated at room temperature overnight. Finally, the gel was stained in 0.2% comassie blue for 2 h and destained in 5% methanol/8% acetic acid till sharp transparent bands indicating calpain activity were visualized in blue background. The gel was scanned using an Odyssey infrared image system (LI-COR Biosciences, Lincoln, NE, USA). The quantification of band intensity was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Intracellular calcium analysis. Fluo-4 AM, a cell-permeant fluorescent calcium indicator, was used to load cells according to the manufacturer's instruction. Briefly, cells were loaded with 1 μM Fluo-4 AM in loading medium (Hanks' balanced salt solution (HBSS)/1% BSA/0.02% pluronic F127/124 μM sulfinpyrazone, pH 7.4) for 30 min at room temperature in the dark, washed twice in indicator-free medium (HBSS/1% BSA/124 μM sulfinpyrazone, pH 7.4), and then incubated in indicator-free medium for another 30 min. Finally, slides were mounted in gelvatol and fluorescent images were acquired using a Sony DXC-979MD 3CCCD video camera connected to Olympus BX40 microscope.

siRNA transfection. siRNA transfection was performed to knockdown calpain 1/2 according to the manufacturer's instruction for TransIT-TKO transfection reagent (Mirus Bio Corporation, Madison, WI, USA). After 24 h of transfection, cells were replaced with fresh growth medium and treated with related reagents.

Western blot analysis. Cells were lysed in RIPA buffer containing protease inhibitors. Equal amounts of proteins were separated on SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 0.1% casein/0.2 × PBS for 1 h, incubated with appropriate primary antibodies for 2 h, then incubated with secondary antibodies conjugated with IRDye 800 (Rockland

Immunochemicals, Gilbertsville, PA, USA) or Alexa Fluor 680 (Invitrogen) for 40 min. After primary and secondary antibodies incubation, membranes were washed four times with TPBS (PBS with 0.1% Tween 20) at 5 min intervals. Immunoblots were visualized using an Odyssey Infrared Imaging System. The quantification of bands intensity was performed using ImageJ software.

Statistical analysis. Data are expressed as mean ± S.D. of three independent experiments and analyzed by one-way ANOVA test using SPSS 14.0 software (SPSS Incorporated, Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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