Molecular Chaperone gp96 Is a Novel Therapeutic Target of Multiple Myeloma

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Abstract

Purpose: gp96 (grp94) is a key downstream chaperone in the endoplasmic reticulum (ER) to mediate unfolded protein response (UPR) and the pathogenesis of multiple myeloma is closely linked to dysregulated UPR. In this study, we aimed to determine the roles of gp96 in the initiation and progression of multiple myeloma in vivo and in vitro.

Experimental Design: We generated a mouse model with overexpression of XBP1s and conditional deletion of gp96 in B-cell compartment simultaneously to identify the roles of gp96 in the development of multiple myeloma in vivo. Using a short hairpin RNA (shRNA) system, we silenced gp96 in multiple human multiple myeloma cells and examined the effect of gp96 knockdown on multiple myeloma cells by cell proliferation, cell-cycle analysis, apoptosis assay, immunohistochemistry, and human myeloma xenograft model. The anticancer activity of gp96 selective inhibitor, WS13, was evaluated by apoptosis assay and MTT assay.

Results: Genetic deletion of gp96 in XBP1s-Tg mice attenuates multiple myeloma. Silencing of gp96 causes severe compromise in human multiple myeloma cell growth through inhibiting Wnt-LRP-survivin pathway. We also confirmed that knockdown of gp96 decreased human multiple myeloma growth in a murine xenograft model. The targeted gp96 inhibitor induced apoptosis and blocked multiple myeloma cell growth, but did not induce apoptosis in pre-B leukemic cells. We have demonstrated that myeloma growth is dependent on gp96 both genetically and pharmacologically.

Conclusions: gp96 is essential for multiple myeloma cell proliferation and survival, suggesting that gp96 is a novel therapeutic target for multiple myeloma.

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Introduction

As an ER chaperone, gp96 (1), also known as grp94 (2), endoplasmin (3), ERP99 (4), and HSP90b1(5), is a parologue of HSP90 and its expression can be induced by the accumulation of misfolded proteins (6). It binds to and hydrolyzes ATP (7–9), and is the most abundant protein in the endoplasmic reticulum (ER) lumen. gp96 is a key downstream chaperone in the ER to mediate unfolded protein response (UPR; refs. 10). UPR is an evolutionally conserved mechanism to maintain protein quality control in the secretory pathway. Accumulation of misfolded proteins in the ER triggers the activation of three well-known pathways including activating transcription factor 6 (ATF6), the double-stranded RNA-activated protein kinase-like ER kinase (PERK), and the spliced form of X box binding protein 1 (XBP1s) to induce the expression of several major ER HSPs including gp96, grp78, and calreticulin to enhance protein folding machinery (11). UPR plays critical roles for plasma cell differentiation as demonstrated by lack of plasma cells in the absence of XBP1 (12–14). Moreover, gp96 is induced more than 10-fold during B-cell activation (4), and it has been shown to participate in the assembly of B-cell receptor complexes through its association with immunoglobin-α (Igα) molecules (15). In addition to the critical roles of UPR in plasma cell differentiation, a picture has recently emerged of the roles of UPR, particularly XBP1s, in myeloma pathogenesis. XBP1s and downstream ER chaperones are consistently upregulated in myeloma cells (16). Most strikingly, transgenic expression of XBP1s in the B-cell compartment of mice results in plasma cell dyscrasia with evidence of increased monoclonal antibodies (“M-spike”), lytic bone lesions, plasmacytosis, and kidney damage (17). However, the roles of individual ER HSPs in myeloma have not been reported.

Using genetic strategies, we recently found that gp96 is an obligate master chaperone for multiple Toll-like receptors (TLR) and integrins (18–21). However, under the
Translational Relevance

Multiple myeloma is an incurable plasma cell neoplasm whose pathogenesis is closely linked to dysregulated unfolded protein response (UPR) in the endoplasmic reticulum (ER). In this study, we demonstrated that 96 is required for mice as well as multiple human multiple myeloma (MM) chronic ER stress conditions. In this study, we addressed biology and for the development of myeloma during It is unclear whether gp96 is required for plasma cell activation, germinal center formation, plasma cell differentiation, and class-switching or affinity maturation (19).

steady-state conditions, gp96 is not required for B-cell activation, germinal center formation, plasma cell differentiation, and class-switching or affinity maturation (19). It is unclear whether gp96 is required for plasma cell biology and for the development of myeloma during chronic ER stress conditions. In this study, we addressed the roles of gp96 in myeloma using XBP1s-transgenic mice as well as multiple human multiple myeloma (MM) cell lines. We demonstrated that gp96 is required for myeloma progression both in vitro and in vivo. Mechanistically, we found that gp96 critically controls the Wnt-LRP-survivin pathway. Our results indicated that gp96 is a novel therapeutic target for multiple myeloma.

Materials and Methods

Mice and cell lines

B-cell–specific gp96-deficient mice and wild-type control littermates have been described (19). B-cell–specific XBP1s-transgenic and gp96-deficient mice were generated by crossing our B-cell–specific gp96-deficient mice with XBP1s transgenic mice. SCID mice were kindly provided by Jennifer Wu (Medical University of South Carolina, Charleston, SC). Mice were bred and maintained according to the established guidelines and an approved protocol by Medical University of South Carolina Institutional Animal Care and Use Committee. Human multiple myeloma cell line RPMI-8226, U266B1, MM.1S, and MM.1R were purchased from American Type Culture Collection. OPM1, JK-6L, and INA-6 were kindly provided by Yubin Kang (Medical University of South Carolina). Multiple myeloma cells were cultured in RPMI medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (FCS; Atlas Biologicals), 55 μmol/L 2-mercaptoethanol (2-ME, Gibco), 1 mmol/L sodium pyruvate (Gibco), 10 mmol/L HEPES (Gibco), and penicillin–streptomycin (Gibco). All cells were cultured in 5% CO2 incubator. Gp96-mutant and WT pre-B-cell lines were provided by Brian Seed (Harvard University, Cambridge, MA).

Reagents

Antibodies used for flow cytometry were obtained from BD Biosciences and eBioscience. Antibodies against LRP6, β-catenin, survivin, c-myc, and caspase-9 were purchased from Cell Signaling Technology, cyclin D1 was obtained from Abcam, and gp96 antibody was bought from Enzo Life Sciences, Inc. Ig levels were determined by a sandwich ELISA kit from Southern Biotechnology Associates. All other chemicals were obtained from Sigma-Aldrich and Fisher Scientific. IL-4 and IL-21 were purchased from PeproTech. WS13, a gp96-specific Hsp90 inhibitor of the purine-scaffold class (22), was synthesized as previously described (23, 24) and the structure was recently reported (25).

Flow cytometry and in vitro plasma cell differentiation

Surface staining of cells and analysis on FACScalibur and FACSVerse (Becton-Dickinson) were done as described (18, 26). B cells were purified from spleens using CD19 magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec). Purified murine splenic B cells were labeled with carboxylfluorescein succinimidyl ester (CFSE), and then stimulated with anti-μ antibody, agonistic CD40 antibody, and IL-21 for 3 days. This was followed by flow cytometric analysis of expression levels of cell surface CD138 by dividing cells.

Viral vectors and transduction

Human gp96 and survivin short hairpin RNA (shRNA) lentiviral vectors as well as control vector were purchased from Open Biosystems. Survivin retroviral vector and empty vector control were obtained from Origene Technologies. Cells were seeded in a 12-well plate and spin-infected with recombinant virus (3,000 rpm, 32°C, 90 minutes) in a desktop centrifuge as reported (26).

Protein extraction and Western blot analysis

Protein extraction and immunoblot were performed as described previously (20). Briefly, cells were washed three times with ice-cold PBS and lysed in radioimmunoprecipitation assay lysis buffer (0.01 mol/L sodium phosphate, pH 7.2, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mmol/L AEBSF, 130 mmol/L bestatin, 14 mmol/L E-64, 0.3 mmol/L aprotinin, and 1 mmol/L leupeptin). Total cell lysates was resolved on denaturing and reducing 10% SDS-PAGE, and the proteins were transferred from the gel onto Immobilon-P membranes. The membrane was blocked with 5% nonfat milk in PBS and then incubated with different antibodies, followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibody. Protein bands were...
visualized by using enhanced chemiluminescent substrate (Pierce) or clarity enhanced chemiluminescence (ECL) substrate (Bio-Rad).

Cell growth and proliferation assays

Cell growth was assessed through the Trypan blue dye exclusion method and MTT cell viability assay according to the manufacturer’s instruction. Cells were seeded at 1 × 10⁵ cells/mL in 96-well plates. The cells were treated with 5 μmol/L gp96-specific inhibitor WS13 or vehicle control and incubated in 5% CO₂ incubator at 37°C for three time points (0, 24, and 72 hours). Plates were read at 570 nm by using iMark microplate absorbance reader (Bio-Rad).

Cell-cycle analyses

A total of 1 × 10⁶ cells were washed twice in cold PBS and fixed with 4 mL of ice-cold 70% ethanol at 4°C overnight. Cells were washed once with PBS and incubated with 40 μg/mL propidium iodide (PI) and 100 μg/mL RNAse for 30 minutes at 37°C in the dark. Cells were analyzed on a FACSCalibur. The percentage of cells in each phase of the cell cycle was quantitated using the FlowJo software (TreeStar).

Apoptosis assay

Apoptosis and cell death were determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining according to the manufacturer’s protocol (Trevigen) and flow cytometry analysis of Annexin V and propidium iodide using Annexin V apoptosis detection Kit (eBioscience).

Human myeloma xenograft model

Severe combined immunodeficient (SCID) mice (8–12 weeks old) were subcutaneously inoculated with 5 × 10⁶ WT control and gp96 knockdown (KD) human myeloma RPMI-8226 cells, respectively. Tumor growth was monitored every week using digital calipers to measure both the longitudinal (a, mm) and transverse (b, mm) diameters. Tumor area (a × b, mm²) was plotted. Mice were also monitored for the following general health indicators: overall behavior, feeding, neuromuscular tone, body weight, and appearance of fur, etc. At the endpoint (8 weeks after tumor inoculation), the primary tumor was also excised and weighed after the mice were sacrificed. Tumor tissues were fixed in 4% formalin or frozen in optimum cutting temperature medium.

Immunohistochemistry

Cryosections of fixed xenograft tumor tissues (5 μm thick) were developed with DAB substrate kit (Vector Labs). Slides were counterstained with hematoxylin before visualization on Zeiss Axio microscope.

Immunofluorescence

Cryosections of xenograft tumor tissues (5 μm thick) were fixed with 4% paraformaldehyde, permeabilized with cold methanol, blocked and stained with gp96 antibody (9G10), and costained with Alexa Fluor 488–conjugated mouse anti-HLA-ABC (BD Biosciences). Images of sections were taken under a fluorescent microscope (Zeiss) and analyzed by AxioVision 4.4 software (Carl Zeiss Micro Imaging).

Microarray analysis

Raw cDNA microarray data of myeloma samples and their corresponding clinical parameters were downloaded from Oncomine database (27). The most specific probe for gp96 was used to represent gene expression levels. Unpaired, two-tailed Student t test was used for comparison of gp96 expression.

Statistical analysis

Error bars represent the SD or the SEM. Student t test and ANOVA were used for statistical analysis. Values of P value less than 0.05 were considered to represent statistically significant differences.

Results

Genetic deletion of gp96 in XBP1s-Tg mice attenuates myeloma

Using CD19cre-mediated deletion of gp96, we previously found that gp96 is dispensable for Ig synthesis, Ig assembly, B-cell differentiation, plasma cell development, and Ig class-switching.(19) It is unclear, however, if gp96 plays important roles in antibody production and myeloma progression during chronic ER stress. To answer this question, we generated B-cell–specific XBP1s-Tg and gp96 knockout (KO) mice (named TKO mice) by crossing B-cell–specific gp96 knockout mice with XBP1s-Tg mice; the latter spontaneously develop multiple myeloma after reaching 40 weeks of age (17). The constitutive expression of XBP1s created a scenario of chronic unresolving ER stress. As expected, without transgenic expression of XBP1s, gp96 deletion does not negatively affect plasma cell number or the level of serum antibodies (Fig. 1; ref. 19). Strikingly, we found that plasma cells (B220⁺/IgM⁺CD138⁺) were significantly reduced in number in both the bone marrow and the spleen of the TKO mice when gp96 was deleted in XBP1s transgenic mouse (Fig. 1A and B). To directly examine the plasma cell differentiation in TKO mice, we stimulated CFSE–labeled splenic B cells from WT, knockout, Tg, and TKO mice with IL-4 alone or the combination of IL-4, anti-μ antibody, agonistic CD40 antibody and IL-21 for 3 days (28). We found that TKO B cells differentiated to plasma cells as efficiently as WT, knockout, or XBP1s Tg B cells based on CD138 expression (Fig. 1C). These data indicated that the reduction of plasma cells in TKO mice is not due to inefficient plasma cell differentiation. Consistent with the
reduced plasma cell number, we also found significant reduction of Ig level (both IgG1 and IgM) in TKO mice (Fig. 2A and B), along with significant reduction of γ-globulin on serum protein electrophoresis (Fig. 2C and D) and decreased Ig deposition in the glomeruli (Fig. 2E). These results suggest that gp96 is required for myeloma progression during chronic ER stress.

gp96 is essential for multiple myeloma cell proliferation and survival

We next mined the Oncomine database (27) to gain a possible clue for the clinical significance of gp96 expression in myeloma. We found that gp96 expression is significantly elevated in multiple myeloma samples from relapsed patients compared with the ones at the initial diagnosis (Fig. 3A; P < 0.05). To further study the mechanism of gp96 dependency by multiple myeloma cells, we next silenced gp96 in human multiple myeloma cell lines using a gp96 shRNA lentiviral vector. We found that silencing of gp96 causes significantly compromised multiple myeloma cell growth. In comparison, a pre-B leukemic cell line, however, does not depend on gp96 for growth (Fig. 3B and C). Cell-cycle analysis of multiple myeloma cells revealed that there were significantly fewer cells in G2 phase and more cells in over-G2 phase upon gp96 knockdown (Fig. 3D). PI stain confirmed that more gp96 knockdown multiple myeloma cells undergo constitutive death (PI+ cells: 60.4% ± 6.62%) than their wild-type counterparts (PI+ cells: 35.3% ± 2.05%; Fig. 3E; P < 0.001). To further determine whether gp96 is required for human multiple myeloma cell growth in vivo, we performed xenograft model by inoculating WT and gp96KD RPMI-8226 multiple myeloma cells to SCID mice.
followed by monitoring tumor growth. Indeed, knockdown of gp96 significantly inhibited multiple myeloma tumor growth (Fig. 3F and G). TUNEL analysis of the xenografts tumor tissues confirmed that the gp96 knockdown multiple myeloma cells underwent massive apoptosis when compared with its wild-type counterpart (Fig. 3H and I).

**gp96 is required for canonical Wnt signaling by controlling LRP6 expression.**

gp96 has recently been implicated in controlling cell surface expression of LRP6 (29). We also demonstrated that there is a strong interaction between LRP6 and gp96 (30). LRP6 is a coreceptor for the cell surface Wnt receptor Frizzled, and is required for canonical Wnt signaling. The Wnt/β-catenin signaling pathway is well known to modulate cell differentiation, proliferation, and apoptosis. The roles of the Wnt pathway in the pathogenesis of multiple myeloma have received increasing attention recently (31-33). To determine whether Wnt/β-catenin signaling defect is responsible for decreasing multiple myeloma cell growth in the absence of gp96, we compared the canonical Wnt signaling between
empty vector (EV) control and gp96 knockdown RPMI-8226 cells by Western blot analysis. We found that the expression of LRP6 is compromised in gp96 knockdown multiple myeloma cells, consistent with the posttranslational chaperoning roles of LRP6 by gp96. Moreover, β-catenin, as well as the Wnt downstream targets, cyclin D1, and c-myc were significantly decreased in gp96 knockdown RPMI-8226 multiple myeloma cells (Fig. 4A). Furthermore, the growth defect of gp96 knockdown RPMI-8226 cells was significantly rescued by GSK3β inhibitors (TWS119 and lithium chloride; Fig. 4B), demonstrating that gp96 promotes myeloma growth via Wnt signaling.

gp96 knockdown compromises expression of survivin and induces mitotic catastrophe in human multiple myeloma cells

We next studied the candidate molecules downstream of Wnt in controlling cell survival. We focused on examining the expression of survivin and caspases in gp96 knockdown multiple myeloma cells. We found that survivin levels were significantly reduced in the gp96 knockdown RPMI-8226 cells, which were associated with an increased level of cleaved caspase-9 (Fig. 4C). The reduction of nuclear survivin in gp96 knockdown cells was further confirmed by immunohistochemistry (Fig. 4D). Survivin is known to play important roles in spindle separation during mitosis (34). As expected, morphologic analysis of the gp96 knockdown multiple myeloma cells revealed a marked tendency toward multi-nucleation (Fig. 4E and F), a sign of mitotic catastrophe (35), which was phenocopied by survivin silencing (Fig. 4E and H). To further address that survivin is the key downstream target of gp96 that is responsible for our observed phenotype, we overexpressed survivin in gp96 knockdown cells. The mitotic arrest was significantly rescued (Fig. 4E and G).

Targeted gp96 inhibitor WS13 inhibits growth and induces death of myeloma cells

HSP90 inhibitors have been shown to be potent and promising anticancer agents (36, 37). Recent studies showed that pan-HSP90 inhibitors such as 17-AAG, Radicicol, and PU-H71 are potential therapeutics for treatment of multiple myeloma (38, 39). However, none of these studies specifically looked into the contribution by different members of HSP90 (cytosolic HSP90, gp96, and mitochondrial TRAP-1) in myeloma (40). We next determined if inhibiting gp96 could be therapeutically beneficial to myeloma without inhibiting cytosolic HSP90. WS13, a gp96-specific HSP90 inhibitor of the purine-scaffold class (22), was synthesized as previously described (23, 24). We treated multiple myeloma cells and pre-B leukemic cells with it. We observed that WS13 induced both apoptosis and necrosis, and inhibited growth of multiple myeloma cells, but did not induce death in pre-B leukemic cells (Fig. 5A and B). Thus, we have demonstrated that myeloma growth is dependent on gp96 both genetically and pharmacologically, strongly indicating that gp96 is a promising therapeutic target in multiple myeloma.
Discussion

UPR plays critical roles in plasma cell differentiation as well as in the pathogenesis of multiple myeloma. XBP1s and downstream ER chaperones are consistently upregulated in myeloma cells in patients (16), inspiring increasing efforts to develop UPR-targeted anti–multiple myeloma therapy (41–43). However, no studies have looked specifically into the roles of individual ER-resident chaperones in multiple myeloma. gp96 is a key downstream chaperone in the ER and mediates UPR, and yet its potential roles in multiple myeloma was unexplored (10). In this study, we selectively deleted gp96 in B cells in XBP1s transgenic mice which overexpress XBP1s in B cells and plasma cells and spontaneously develop multiple myeloma after reaching advanced age (17). We found that gp96 is required for maintenance of plasma cells in this model and it is a key driver for development of multiple myeloma. Moreover, we have demonstrated both genetically and pharmacologically that targeted inhibition of gp96 resulted in significant compromise in multiple myeloma cell growth and death that was mediated at least in part by the loss of canonical Wnt pathway (Figs. 1–3). It would be interesting to test the roles of gp96 in other myeloma models such as a transgenic mouse model that expresses myc transgene in a activation-induced deaminase (AID)-dependent manner (44). This is important given the limitation of XBP1s transgenic model in faithfully representing human myeloma (45).

Our study has added significantly to the emerging role of Wnt signaling in multiple myeloma (31–33). Human multiple myeloma cells appear to have hallmarks of active Wnt signaling by overexpressing β-catenin which promotes proliferation of multiple myeloma (46). Furthermore, blocking β-catenin with small-molecule inhibitors, AV-65 (33) or PKF115-584 (31, 32) specifically inhibits multiple myeloma cell proliferation. Paradoxically, higher expression of Wnt signaling inhibitor, Dickkopf1 (Dkk1) in bone marrow stromal cells can promote myeloma growth (47). Still other studies demonstrated that increasing Wnt signaling did not significantly affect the proliferation of multiple myeloma cells (48, 49). These studies suggest that Wnt signaling can

Figure 5. Selective gp96 inhibitor WS13 inhibits growth of human multiple myeloma cells and induces apoptosis. A, pre-B leukemic cells, RPMI-8226, MM.1S, MM.1R, JK-6L, INA-6, OPM1, and U266B1 multiple myeloma cells, were treated with 5 μmol/L WS13 or vehicle control for 24 hours followed by flow cytometry analysis for necrotic cells (PI+/Annexin V+) or apoptotic cells (PI+Annexin V+). B, pre-B leukemic cells RPMI and multiple human multiple myeloma cells were treated with 5 μmol/L WS13 or vehicle control for 24, 72, and 120 hours followed by quantification of live cells by MTT assay. Error bars indicate SD. * , P < 0.05; **, P < 0.01; ***, P < 0.001 (t test).
play both positive and negative roles in the progression of multiple myeloma, likely due to the difference in the underlying pathogenesis. Our study has firmly linked the roles of gp96 with its ability to chaperone Wnt coreceptor LRP6. We showed that gp96 is required for multiple myeloma development in XBP1s transgenic mouse model whose underlying pathogenesis is dysregulated UPR. To illustrate the importance of cell type-selective roles of gp96, we found that silencing of gp96 causes significant compromise in the growth of multiple myeloma cells but not pre-B leukemic cells (Fig. 3). Thus, it is possible that multiple myeloma cells, with hallmarks of ER stress, are uniquely dependent on gp96. The explanation could be that multiple myeloma cells have a particularly stressed ER due to heightened production of Ig. When rate of protein synthesis is increased, UPR leads to increased level of HSPs including gp96 to accelerate protein folding. In the absence of gp96, the folding capacity of the ER in multiple myeloma cells is exceeded by chronic accumulation of misfolded proteins. In addition, loss of gp96 causes collapse of canonical Wnt signaling. Therefore, gp96 regulates multiple myeloma via both UPR and Wnt pathways. Our work thus suggests that Wnt pathway is under the regulation of UPR. The interplay between Wnt pathway and UPR in multiple myeloma deserves further study. Although speculative, it might also be fruitful to look at the combination of targeted therapy against both Wnt and UPR pathway for the treatment of multiple myeloma in the future.

Cancer cell metabolism is necessarily abnormal to accommodate the internal and external pressures which are secondary to excessive growth: nutrient insufficiency, free radical generation, and cellular machinery overloaded with accelerated DNA replication and protein production. gp96 stands at the intersection of causative and responsive in regard to such changes. Increased protein production upregulates gp96, making it an important compensatory response to malignant growth, and acting to protect the cell from ER stress. However, upregulated gp96 may also actively support enhanced growth signaling in the form of the canonical Wnt pathway, a commonly upregulated signaling cascade in cancer. Interestingly, survivin has been reported to be a target of Wnt signaling (33). Survivin is normally absent in healthy cells, appearing only during regulated passage through G2-M. Overexpression of survivin is correlated with multidrug resistance in multiple myeloma (50). Here, we have demonstrated that gp96 knockdown induces mitotic catastrophe and apoptosis in human multiple myeloma cells, which is characterized by loss of survivin and appearance of multi-nucleated cells (Fig. 4). Our data allow us to propose a model of the roles of gp96 in multiple myeloma cell survival (Fig. 6). We believe that gp96 is an especially important mediator of malignant growth due to its dual roles of absorbing the ER stress of cancer-driven protein production and also instigating further Wnt-survivin growth signaling.

In summary, we have uncovered that both murine and human myeloma cells are ostensibly dependent on gp96 for growth in vivo and in vitro. Mechanistically, we have linked the roles of gp96 in promoting multiple myeloma to its function in chaperoning Wnt-LRP-survivin pathway. Thus, gp96-targeted inhibitors such as WS13 may prove to be a novel therapeutic against multiple myeloma in the future.

Disclosure of Potentials Conflicts of Interest
S.Z. Usmani has a commercial research grant from Celgene, Millennium, Onyx, honoraria from speakers’ bureau from Celgene and Onyx. G. Chiosis is employed in the Board of directors (other than primary affiliation; e.g., consulting) of Samus Therapeutics and has ownership interest (including patents) in Samus Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Liu
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