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Caspase-, cathepsin-, and PERK-dependent regulation of MDA-7/IL-24-induced cell killing in primary human glioma cells

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Abstract

Melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL-24) is a novel cytokine displaying selective apoptosis-inducing activity in transformed cells without harming normal cells. The present studies focused on defining the mechanism(s) by which a GST-MDA-7 fusion protein inhibits cell survival of primary human glioma cells *in vitro*. GST-MDA-7 killed glioma cells with diverse genetic characteristics that correlated with inactivation of ERK1/2 and activation of JNK1-3. Activation of JNK1-3 was dependent on protein kinase R-like endoplasmic reticulum kinase (PERK), and GST-MDA-7 lethality was suppressed in PERK^{-/-} cells. JNK1-3 signaling activated BAX, whereas inhibition of JNK1-3, deletion of BAX, or expression of dominant-negative caspase-9 suppressed lethality. GST-MDA-7 also promoted a PERK-, JNK-, and cathepsin B-dependent cleavage of BID; loss of BID function promoted survival. GST-MDA-7 suppressed BAD and BIM phosphorylation and heat shock protein 70 (HSP70) expression. GST-MDA-7 caused PERK-dependent vacuolization of LC3-expressing endosomes whose formation was suppressed by incubation with 3-methyladenine, expression of HSP70 or BiP/GRP78, or knockdown of ATG5 or Beclin-1 expression but not by inhibition of the JNK1-3 pathway. Knockdown of ATG5 or Beclin-1 expression or overexpression of HSP70 reduced GST-MDA-7

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lethality. Our data show that GST-MDA-7 induces an endoplasmic reticulum stress response that is causal in the activation of multiple proapoptotic pathways, which converge on the mitochondrion and highlight the complexity of signaling pathways altered by *mda-7/IL-24* in glioma cells that ultimately culminate in decreased tumor cell survival.

Introduction

In the United States, glioblastoma multiforme (GBM) is diagnosed in ~20,000 patients per annum. High-grade tumors, such as anaplastic astrocytoma and GBM, account for the majority of astrocytic tumors (1). Even under ideal circumstances, in which essentially all of the tumor can be surgically removed and the patients are maximally treated with radiation and chemotherapy, the mean survival of this disease is only extended from 2 to 3 months to 1 year (1). These statistics emphasize the need to develop therapies against this devastating and invariably fatal disease.

The *mda-7* gene [recently renamed interleukin (IL)-24] was isolated from human melanoma cells induced to undergo terminal differentiation by treatment with fibro-blast IFN and mezerein (2). The protein expression of MDA-7/IL-24 is decreased in advanced melanomas, with nearly undetectable levels in metastatic disease (2–4). This novel cytokine is a member of the IL-10 gene family (5–12). Enforced expression of MDA-7/IL-24, by use of a recombinant adenovirus Ad.*mda-7*, inhibits the growth and kills a broad spectrum of cancer cells, without exerting deleterious effects in normal human epithelial or fibroblast cells (9–14). Considering its potent cancer-specific apoptosis-inducing ability and tumor growth-suppressing properties in human tumor xenograft animal models, *mda-7/IL-24* was evaluated in a phase I clinical trial in patients with advanced cancers (10, 11, 15). This study indicated that Ad.*mda-7* injected intratumorally was safe, and with repeated injection, significant clinical activity was evident.

The apoptotic pathways by which Ad.*mda-7* causes cell death in tumor cells are not fully understood; however, current evidence suggests an inherent complexity and an involvement of proteins important for the onset of growth inhibition and apoptosis, including BCL-x_L, BCL-2, BAX, and APO2/TRAIL (9–14). In melanoma cell lines but not in normal melanocytes, Ad.*mda-7* infection induces a significant decrease in both BCL-2 and BCL-x_L levels, with only a modest up-regulation of BAX and BAK expression (16). These data support the hypothesis that Ad.*mda-7* enhances the ratio of proapoptotic to antiapoptotic proteins in cancer cells, thereby facilitating induction of apoptosis (9–14, 16, 17). The ability of Ad.*mda-7* to induce apoptosis in DU145 prostate cancer cells, which does not produce BAX, indicates that MDA-7/IL-24 can also mediate apoptosis in tumor cells by a BAX-independent pathway (9–12). In prostate cancer cells, overexpression of either BCL-2 or BCL-x_L protects cells from Ad.*mda-7*-induced toxicity in a cell type-dependent fashion (18). Thus, MDA-7/IL-24 lethality seems to occur by multiple distinct pathways in different cell types. More recently, MDA-7/IL-24 toxicity has been linked to alterations in endoplasmic reticulum (ER) stress signaling (19). In these studies, MDA-7/IL-24 physically associates with BiP/GRP78 and inactivates the protective actions of this ER chaperone protein. In addition to virus-administered *mda-7/IL-24*, delivery of this cytokine as a bacterially expressed GST fusion protein, GST-MDA-7, retains cancer-specific killing, selective ER localization and induces similar signal transduction changes in cancer cells. We have noted that high concentrations of GST-MDA-7 or infection with Ad.*mda-7* kill rodent and human glioma cells (20–23). However, the precise mechanisms by which Ad.*mda-7* and GST-MDA-7 modulate cell survival in nonestablished human glioma cells are presently unknown.

The ability of MDA-7/IL-24 to modulate cell signaling processes in transformed cells has been investigated by several groups (20, 22, 24–27). Our laboratories have shown that Ad.*mda-7* kills melanoma cells and established glioma cells in part by promoting p38 mitogen-activated protein kinase–dependent activation of the growth arrest and DNA damage-inducible genes, including GADD153, GADD45, and GADD34 (20, 24). Other groups have argued that inhibition of phosphatidylinositol-3-kinase signaling, but not ERK1/2 signaling, modestly promotes Ad.*mda-7* lethality in breast and lung cancer cells (26, 27). Prior work by our groups has shown, using bacterially synthesized GST-MDA-7 protein, that in the 0.25 to 2.0 nmol/L concentration range GST-MDA-7 primarily causes growth arrest with little cell killing, whereas at ~20-fold greater concentrations this cytokine causes profound growth arrest and tumor cell death (20, 23, 24, 28). The toxicity of low nanomolar GST-MDA-7 concentrations were elevated by multiple agents that generate reactive oxygen species, which correlates with prolonged activation of the JNK1/2 pathway and associates with tumor cell killing (16, 24). Using primary human GBM isolates cultured *in vitro* as well as transformed fibroblasts lacking expression of specific proapoptotic proteins, we currently examined the effect of GST-MDA-7 on cell viability with a focus on elucidating the molecular mechanisms by which GST-MDA-7 enhances cell death.

Materials and Methods

Materials

Transformed protein kinase R–like ER kinase (PERK) $-/-$ cells were a kind gift from Dr. D. Ron (Skirball Institute, New York University School of Medicine). Transformed cathepsin B $-/-$ fibroblasts and matched wild-type (WT) fibroblasts were kindly supplied by Drs. C. Peters and T. Reinheckel (Medizinische Universitaetsklinik Freiburg) and Dr. P. Saftig (Christian-Albrechts-Universitaet Kiel; ref. 29). Plasmids expressing dominant-negative PERK (dnPERK), BiP/GRP78, and LC3-GFP were kindly supplied by Dr. A. Diehl (University of Pennsylvania), Dr. A. Lee (University of California-Los Angeles), and Dr. S. Spiegel (Virginia Commonwealth University). Short hairpin RNA constructs targeting *ATG5* (pLVTHM/ATG5) were a generous gift from Dr. Yousefi (Department of Pharmacology, University of Bern); *Beclin-1* (pSRP-Beclin-1) was kindly provided by Dr. Yuan (Department of Cell Biology, Harvard Medical School; refs. 30–33). Antibody reagents, kinase inhibitors, caspase inhibitors, cell culture reagents, primary human GBM cells, and noncommercial recombinant adenoviruses have been described previously (18, 21, 23).

Methods

Generation of Ad.*mda-7* and Synthesis of GST-MDA-7—Recombinant type 5 adenovirus to express MDA-7 (Ad.*mda-7*), control (CMV vector), or control (β -galactosidase) were generated using recombination in HEK293 cells as described in refs. 19–21, 25–27, 31.

Cell Culture and *In vitro* Exposure of Cells to GST-MDA-7 and Drugs—All established cell lines were cultured at 37°C [5% (v/v) CO₂] *in vitro* using RPMI supplemented with 5% (v/v) FCS and 10% (v/v) nonessential amino acids. Primary human glioma cells were subcultured in 2% (v/v) FCS to prevent growth of contaminating rodent fibroblasts for 1 week before *in vitro* analyses, after which cells were cultured in 5% (v/v) FCS. For short-term cell killing assays and immunoblotting, cells were plated at a density of 3×10^3 per cm² and 36 h after plating were treated with MDA-7/IL-24 and/or various drugs as indicated. *In vitro* small-molecule inhibitor treatments were from a 100 mmol/L stock solution of each drug and the maximal concentration of vehicle (DMSO) in medium was 0.02% (v/v). For adenoviral infection, cells were infected 12 h after plating and the

expression of the recombinant viral transgene was allowed to occur for 24 h before any additional experimental procedure. Cells were not cultured in reduced serum medium during any study.

Cell Treatments, SDS-PAGE, and Western Blot Analysis—Cells were treated with various GST-MDA-7 concentrations as indicated in the figure legends. For SDS-PAGE and immunoblotting, cells were lysed either in a nondenaturing lysis buffer and prepared for immunoprecipitation as described in refs. 24, 28 or in whole-cell lysis buffer [0.5 mol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1% β -mercaptoethanol, 0.02% bromophenol blue], and the samples were boiled for 30 min. After immunoprecipitation, samples were boiled in whole-cell lysis buffer. The boiled samples were loaded onto 10% to 14% SDS-PAGE and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22- μ m nitrocellulose and immunoblotted with indicated primary antibodies against the different proteins.

Recombinant Adenoviral Vectors: Infection *In vitro*—We generated and purchased previously noted recombinant adenoviruses to express constitutively activated and dominant-negative AKT and MEK1 proteins, dominant-negative caspase-9, heat shock protein 70 (HSP70), XIAP, c-FLIP-s, CRM A, and BCL-x_L (Vector Biolabs). Cells were infected with these adenoviruses at approximate multiplicities of infection of 50. Cells were incubated for 24 h to ensure adequate expression of transduced gene products before drug exposures.

Detection of Cell Death by Trypan Blue, Hoechst, Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling, and Flow Cytometric Assays

—Cells were harvested by trypsinization with trypsin/EDTA for ~10 min at 37°C. As some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1,500 rpm for 5 min. The pooled cell pellets were resuspended and mixed with trypan blue dye. Trypan blue stain, in which blue dye incorporating cells were scored as being dead, was done by counting of cells using a light microscope and a hemacytometer. Five hundred cells from randomly chosen fields were counted and the number of dead cells was counted and expressed as a percentage of the total number of cells counted. For confirmatory purposes, the extent of apoptosis was evaluated by assessing Hoechst-stained and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling–stained cytopsin slides under fluorescent light microscopy and scoring the number of cells exhibiting the “classic” morphologic features of apoptosis and necrosis. For each condition, 10 randomly selected fields per slide were evaluated, encompassing at least 1,500 cells. Alternatively, the Annexin V/propidium iodide (PI) assay was carried to determine cell viability out as per the manufacturer’s instructions (BD PharMingen) using a Becton-Dickinson FACScan flow cytometer.

Preparation of S-100 Fractions and Assessment of Cytochrome c Release

Cells were harvested after GST-MDA-7 treatment by centrifugation at 600 rpm for 10 min at 4°C and washed in PBS. Cells ($\sim 1 \times 10^6$) were lysed by incubation for 3 min in 100 μ L lysis buffer containing 75 mmol/L NaCl, 8 mmol/L Na₂HPO₄, 1 mmol/L NaH₂PO₄, 1 mmol/L EDTA, and 350 μ g/mL digitonin. The lysates were centrifuged at 12,000 rpm for 5 min, and the supernatant was collected and added to an equal volume of 2 $\times$ Laemmli buffer. The protein samples were quantified and separated by 15% SDS-PAGE.

Plasmid Transfection—Plasmid DNA (0.5 μ g/total plasmid transfected) was diluted into 50 μ L RPMI that lacked supplementation with FBS or penicillin-streptomycin. LipofectAMINE 2000 reagent (1 μ L; Invitrogen) was diluted into 50 μ L growth medium

that lacked supplementation with FBS or penicillin-streptomycin. The two solutions were then mixed together and incubated at room temperature for 30 min. The total mix was added to each well (4-well glass slide or 12-well plate) containing 200 μ L growth medium that lacked supplementation with FBS or penicillin-streptomycin. The cells were incubated for 4 h at 37°C, after which time the medium was replaced with RPMI containing 5% (v/v) FBS and 1 $\times$ penicillin-streptomycin.

Microscopy for Acidic Endosomes and LC3-GFP Expression—Transfected cells were pretreated with 3-methyl-adenine (3MA; 5 mmol/L; Sigma) 30 min before GST-MDA-7 exposure and then cultured for 12 to 48 h. Cells were then stained with LysoTracker Red Dye (Invitrogen) at the indicated time points for 20 min. LysoTracker Red Dye-stained cells were visualized immediately after staining on a Zeiss Axiovert 200 microscope using the rhodamine filter. LC3-GFP-transfected cells were visualized at the indicated time points on the Zeiss Axiovert 200 microscope using the FITC filter.

Data Analysis—Comparison of the effects of various treatments was done using one-way ANOVA and a two-tailed Student's *t* test. Differences with $P < 0.05$ were considered statistically significant. Experiments shown are the means of multiple individual points from multiple experiments ($\pm$ SE).

Results

Initial experiments focused on defining potential mechanisms by which GST-MDA-7 promotes cell killing in four independent primary human glioma cell populations: GBM5, GBM6, GBM12, and GBM14 (23). Treatment of GBM5, GBM6, GBM12, and GBM14 cells with GST-MDA-7 was first noted to induce cell killing ~48 h after exposure and caused significant cell killing within 72 h (Fig. 1A; Table 1; Supplementary Fig. S1).⁹ Cell killing was blocked by a pan-caspase inhibitor and an inhibitor of caspase-9 but not by an inhibitor of caspase-8. Cell killing correlated with increased release of cytochrome *c* into the cytosol of GBM6 cells and with the cleavage of caspase-3 (Fig. 1A, *top*). In GBM6 cells, GST-MDA-7 treatment caused inactivation of ERK1/2 that correlated with dephosphorylation of BAD S112 and dephosphorylation and increased expression of BIM (Fig. 1B). In GBM6 cells, GST-MDA-7 treatment caused activation of JNK1-3 that was causal in the activation of BAX and the cleavage of BID and caspase-3 (Fig. 1B). However, the decrease in BCL-x_L protein expression caused by GST-MDA-7 treatment was noted to be JNK independent. In all four glioma isolates, inhibition of caspase-8 function (IETD; expression of CRM A or c-FLIP-s) did not alter GST-MDA-7 lethality, whereas inhibition of caspase-9 function (LEHD; expression of dominant-negative caspase-9 or XIAP or BCL-x_L) significantly reduced GST-MDA-7 lethality as judged by cells becoming PI positive or retaining trypan blue dye (Fig. 1C; Supplementary Fig. S2; data not shown).⁹ GST-MDA-7 lethality correlated with caspase-9-dependent cleavage of pro-caspase-3 (Fig. 1C, *top inset*).

To further investigate mechanisms of GST-MDA-7-induced mitochondrial dysfunction, we used SV40 large T antigen transformed mouse embryonic fibroblasts (MEF) devoid of expression of defined proapoptotic genes. Loss of BIM or BAK function had a modest but significant effect on GST-MDA-7 lethality, whereas loss of BAX and loss of BAX and BAK expression profoundly reduced toxicity (Fig. 1D). In agreement with data in Fig. 1B, genetic manipulation to delete expression of BID also significantly reduced GST-MDA-7 toxicity. Loss of BAX and BAK function or loss of BID function reduced the ability of GST-MDA-7 to promote cytochrome *c* release into the cytosol (Fig. 1C, *top immunoblotting*). In

⁹Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

agreement with data showing that BAX activation and BID cleavage were JNK dependent, inhibition of JNK1-3 with JNK inhibitory peptide treatment suppressed the toxicity of GST-MDA-7 in GBM6 cells from $28.5 \pm 1.3\%$ above the GST control value with vehicle treatment to $7.6 \pm 0.8\%$ above the GST control value ($\pm$ SE; $n = 3$). Collectively, these findings argue that GST-MDA-7 promoted activation of multiple proteins, which act to induce mitochondrial dysfunction, and that activation of the intrinsic mitochondrial pathway represents an important apoptotic mechanism for this cytokine in transformed cells.

In Fig. 1, we noted that BID function, but not caspase-8 function, correlated with GST-MDA-7-induced lethality. BID is a substrate for both caspase-8 and cathepsin proteases, and in glioma cells, cathepsin enzymes are overexpressed and play a key role in tumor invasion and angiogenesis (29, 34). GST-MDA-7 toxicity was nearly abolished by the loss of cathepsin B expression comparing appropriate matched immortalized rodent fibroblast cells, of a different lineage to those used in Fig. 1, which correlated with a reduction in the GST-MDA-7-induced release of cytochrome *c* into the cytosol of these cells (Fig. 2A). Combined inhibition of caspase-9 and cathepsin function was required to suppress GST-MDA-7 lethality in transformed fibroblasts and in GBM6 and GBM12 cells (Table 2). Loss of cathepsin B function suppressed the GST-MDA-7-induced degradation of BID and caspase-3 in transformed fibroblasts, and inhibition of cathepsin B function suppressed the GST-MDA-7-induced degradation of BID in GBM6 cells [Fig. 2B, (i) and (ii)]. The cleavage of p43 cathepsin B after GST-MDA-7 treatment was suppressed by inhibition of JNK1-3 [Fig. 2B, (ii)]. Collectively, these findings suggest that GST-MDA-7 induces multiple parallel proapoptotic pathways in transformed cells that converge to cause mitochondrial dysfunction: a JNK1-3-dependent activation of BAX; a JNK1-3-dependent activation of cathepsin B, leading to a cathepsin B-dependent cleavage of BID; and increased activity of BAD and BIM.

MDA-7/IL-24 has been suggested to promote ER stress signaling and cell death by binding to and inactivating the PERK-binding protein BiP/GRP78 (19). Treatment of transformed fibroblasts that lacked expression of PERK (PERK^{-/-}) with GST-MDA-7 caused significantly less cell killing than observed in their isogenic matched WT counterparts (Fig. 3A). This correlated with reduced release of cytochrome *c* into the cytosol of GST-MDA-7 treated PERK^{-/-} cells; cytochrome *c* release into the cytosol was JNK dependent (Fig. 3B, *top blotting*). Of note, this observation was the opposite to treating these cells with an established inducer of ER stress, thapsigargin (Supplementary Fig. S3).⁹ Surprisingly, based on known downstream targets of PERK signaling, expression of a dominant-negative eIF2 α S51A protein only modestly modified the survival response of GBM6 cells treated with GST-MDA-7 (Supplementary Fig. S4).⁹ GST-MDA-7-induced BID cleavage, cathepsin B cleavage, suppression of BCL-x_L expression, inhibition of ERK1/2 phosphorylation, and increased eIF2 α S51 phosphorylation in transformed fibroblasts were PERK dependent (Fig. 3C).

Considering published reports indicating that ER stress signaling is linked to the activation of the JNK pathway, and our present studies showing that GST-MDA-7-induced toxicity is JNK1-3 dependent and that inhibition of JNK signaling block cytochrome *c* release, we determined whether loss of PERK expression altered the activation of JNK1-3 following GST-MDA-7 exposure (35–38). Treatment of WT-transformed fibroblasts with GST-MDA-7 promoted JNK1/2 activation, predominantly JNK1, which was causal in cell killing (Fig. 3C). In PERK^{-/-} cells, JNK1/2 was very weakly activated by GST-MDA-7, whereas, of note, loss of cathepsin B function did not alter JNK1/2 activation. Hence, GST-MDA-7 induces a PERK-dependent form of ER stress that promotes JNK pathway-dependent activation of BAX and mitochondrial dysfunction as well as promoting a JNK-dependent

activation of cathepsin B that acts to cleave and activate BID, thereby likely promoting further BAX activation and mitochondrial dysfunction.

ER stress-induced cell killing can also be mediated by caspase-2 and caspase-4 that can cause mitochondrial dysfunction as well as initiate cell killing directly (39). Knockdown via small interfering RNA (siRNA) of caspase-2 or caspase-4 expression in GBM6 glioma cells partially, albeit significantly, reduced GST-MDA-7 toxicity in this cell line (Fig. 3D; see also *inset on top right*, confirming siRNA knockdown); GST-MDA-7 caused pro-caspase-2 and pro-caspase-4 cleavage that was JNK dependent (Fig. 3D, *bottom right*).

Because GST-MDA-7 promotes ER stress signaling, we investigated whether cell killing was associated with lysosomal vacuolization and whether any processes known to be associated with autophagy occurred. Treatment of transformed MEFs with GST-MDA-7 caused vacuolization of acidic compartments within 12 h, as judged by Lyso-tracker Red staining, an effect that was not observed in PERK^{-/-} cells at either 12 or 24 h after GST-MDA-7 treatment [Fig. 4A, (*i*); data not shown]. As noted previously, 24 h after GST-MDA-7 exposure, relatively little cell induction of cell killing was observed (data not shown). In MEFs expressing a bona fide dominant-negative form of one downstream substrate of PERK, eIF2 α S51A, vacuolization of acidic compartments was observed at 12 and 24 h, albeit to a lesser extent than that noted in WT cells. The acidic compartment vacuolization effect in transformed MEFs, and in GBM6 and U251 cells, was suppressed by a nonspecific inhibitor of autophagy, 3MA, suggestive that cells may be undergoing autophagy [Fig. 4A, (*ii*)]. GST-MDA-7-induced vacuolization of acidic compartments was not blocked by inhibition of JNK1-3 or p38 mitogen-activated protein kinase (data not shown).

Based on findings showing 3MA-dependent GST-MDA-7-induced vacuolization of acidic compartments, we determined whether the vacuoles also contain a marker for autophagy, LC3. Cells were transfected with a GFP-tagged form of LC3, treated with GST-MDA-7, and the vacuolization of LC3-GFP into punctuate bodies was determined by fluorescent microscopy. In U251 cells, GST-MDA-7 caused vacuolization of LC3-GFP within 24 h, effects that were also 3MA dependent (Fig. 4B; Table 3). Identical data to that in U251 cells were obtained in GBM6 cells with respect to GST-MDA-7- and 3MA-dependent vacuolization of LC3-GFP (Fig. 4B; Table 3). Transfection with GFP alone did not generate punctuate bodies after GST-MDA-7 treatment (data not shown).

Considering that GST-MDA-7-induced cell killing appeared to cause vacuolization, which also contained putative autophagic vacuoles, we explored whether these events were dependent on the function of PERK. GST-MDA-7 induced punctuate staining of GFP-LC3 vacuoles in U251 and GBM6 cells within 24 h that were blocked by transient transfection of dnPERK [Fig. 4C, (*i*); Table 3]. Based on these findings, we determined whether knockdown of ATG5 or Beclin-1, proteins that are known to play a regulatory role in autophagy, altered GST-MDA-7-induced vacuole formation. The ATG12-ATG5 and the ATG8 (LC3)-PE conjugation systems are interdependent, and a disruption in one system has a direct negative effect on the autophagic process (30–33). Beclin-1 is a functional component of the lipase signaling complex, which is essential for the induction of autophagy (30–33). Therefore, perturbation of the levels of ATG5 or Beclin-1 should result in reduced autophagy and the attenuation of the biological effects of GST-MDA-7. To test this, RNA interference was used to specifically suppress ATG5 or Beclin-1 protein levels in tumor cells. Knockdown of ATG5 or Beclin-1 expression significantly suppressed GST-MDA-7-induced GFP-LC3 vacuolization in U251 cells [Fig. 4C, (*ii*); Table 4]. In agreement with these findings, treatment of U251 cells with GST-MDA-7 caused increased expression of ATG5 and Beclin-1 within 24 h as well as the cleavage of endogenous LC3 protein [Fig. 4C,

(ii)]. In transformed fibroblasts, treatment with GST-MDA-7 also caused increased expression of ATG5, a more modest increase in Beclin-1 levels, and modification of endogenous LC3 protein, effects that were abolished in PERK^{-/-} cells (Fig. 4D). Collectively, these data argue that GST-MDA-7 causes an initial autophagic response in human glioma cells and transformed rodent fibroblasts *in vitro*.

Based on the findings in Fig. 4, we determined whether modulation of PERK function or ER stress signaling altered GBM cell survival after GST-MDA-7 exposure. Overexpression of the MDA-7/IL-24 and PERK-binding protein BiP/ GRP78 significantly suppressed LC3-GFP vacuolization after GST-MDA-7 exposure and suppressed GST-MDA-7 toxicity by $64 \pm 4.9\%$ ($\pm$ SE; $n = 3$; Fig. 5A, *top*), that is, overexpressed exogenous BiP/GRP78 bound to GST-MDA-7 inside the cell, and thus lowered the free intracellular concentration of GST-MDA-7, reducing the overall level of cell killing.

In concordance with our data showing that 3MA blocked GST-MDA-7-induced LC3-GFP vacuolization, treatment of GBM6 and U251 cells with 3MA also significantly reduced the toxicity of GST-MDA-7 (Fig. 5A, *bottom*). Knockdown of either Beclin-1 or ATG5 expression in U251 and/or GBM6 cells suppressed GST-MDA-7 lethality (Fig. 5B; Supplementary Fig. S5).⁹ Collectively, these results suggest a direct link between toxicity induction by GST-MDA-7 and promotion of autophagy by GST-MDA-7 in human GBM cells.

The induction of BiP/GRP78 expression is considered as one classic sign of ER stress. GST-MDA-7 rapidly increased expression of BiP/GRP78, and in a delayed fashion decreased expression of the protective protein HSP70, in U251 and GBM6 cells (Fig. 5C). Overexpression of HSP70 reduced GST-MDA-7-induced LC3-GFP vacuolization, particularly at times where HSP70 expression had been suppressed by GST-MDA-7 treatment, and overexpression of HSP70 suppressed GST-MDA-7 toxicity (Figs. 5D and 6A). Overexpression of HSP70 did not, however, abolish GST-MDA-7 toxicity. Collectively, our data suggest that GST-MDA-7 induces parallel cytotoxic death signals and cytoprotective survival signals in GBM cells.

Discussion

Previous studies confirm that GST-MDA-7 reduces proliferation and causes tumor cell-specific and transformed cell-specific killing and radiosensitization in malignant glioma and breast cancer cells. However, although JNK signaling plays a key role in radiation-enhanced killing by *mda-7/IL-24* of tumor cells, the precise signaling pathways provoked by GST-MDA-7 as a single agent and casually related to its cancer-specific cell killing effects in human glioma cells are not well understood (19, 22, 23, 28). The studies in this research were designed to shed light on these issues and define how primary human glioma cells respond to GST-MDA-7 exposure and how, mechanistically, alterations in multiple signaling pathways affect their cell viability.

A GST-MDA-7 concentration that caused profound toxicity ~72 h after exposure in glioma cells correlated with strong activation of JNK1-3. This treatment nearly abolished ERK1/2 signaling. Multiple studies using a variety of cytokine and toxic stimuli document that JNK1-3 activation in astrocytes, neurons, and transformed versions of these cells can trigger cell death (37). The balance between the readouts of ERK1/2 and JNK1-3 signaling may represent a key homeostatic mechanism that regulates cell survival versus cell death processes (38). GST-MDA-7-induced JNK1-3 signaling was PERK dependent and causal in BAX activation, with loss of BAX expression reducing GST-MDA-7-induced cell killing. GST-MDA-7-induced suppression of ERK1/2 signaling was also found to be PERK

dependent. These findings argue that a form of ER stress signaling may be a primary mediator of GST-MDA-7-induced toxicity in primary malignant glioma cells (Fig. 6B).

In some cell types, such as LNCaP prostate cancer cells, the toxicity of Ad.mda-7, either as an individual agent or when combined with a reactive oxygen species-inducing treatment, such as ionizing radiation exposure, has been linked to changes in mitochondrial function (18). This infection results in altered ratios in the expression of proapoptotic BH3 domain-containing proteins, such as BAX, and antiapoptotic proteins, such as BCL-2 and BCL-x_L, with the subsequent release of cytochrome *c* into the cytosol followed by activation of caspase-9 and caspase-3 (9–12). However, in other cell types, such as DU145, which lack expression of BAX, Ad.mda-7 is an even more potent inducer of tumor cell death than is observed in LNCaP cells. This observation suggests that MDA-7/IL-24 must simultaneously induce multiple pathways of mitochondrial dysfunction to provoke tumor cell killing (18). In one study, MDA-7/IL-24 lethality was shown to be mediated by CD95-caspase-8 signaling, indicating that the extrinsic pathway was activated (9, 10, 12). In these contexts, MDA-7/IL-24-induced cancer cell toxicity has been ascribed to expression and activity changes in multiple proapoptotic and antiapoptotic proteins that may occur in a cancer or transformed cell type-specific manner.

In primary human GBM cells and transformed rodent fibroblasts, GST-MDA-7 promoted cell killing by multiple overlapping mechanisms, which all converged on promoting mitochondrial dysfunction; however, activation of death receptor-caspase-8 signaling was not involved in any GST-MDA-7-stimulated death processes in these cells. Knockdown of apoptosis-inducing factor expression did not alter GST-MDA-7 lethality in U251 cells.¹⁰ GST-MDA-7 activated a PERK-JNK-BAX pathway to initiate mitochondrial dysfunction. Although cell killing was reduced in PERK^{-/-} cells, GST-MDA-7 toxicity was still evident and other ER stress regulatory proteins as well as other sensors of the unfolded protein response (e.g., activating transcription factor 6, inositol-requiring enzyme 1, PKR, HRI, and GCN2) may mediate the toxic response of GST-MDA-7 (40). Prior studies have implicated MDA-7/IL-24 as a protein that associates with and activates PKR (41). As PERK and PKR are proteins with structural similarities, it is possible that PKR and PERK represent MDA-7/IL-24 targets in the regulation of eIF2 α phosphorylation and transformed cell survival. Matsuzawa et al. implicated a TRAF2-ASK1-JNK cascade downstream of inositol-requiring enzyme 1 in ER stress responses in multiple cell types, and based on our data, PERK-dependent signaling could also feed into this survival regulatory process (36).

In addition to the regulation of BAX, GST-MDA-7 also caused PERK- and JNK-dependent activation of cathepsin B, which resulted in a caspase-8-independent cleavage of BID that also acted to promote mitochondrial dysfunction. Cathepsin-mediated cell death processes can also occur independently of mitochondrial dysfunction (42, 43). ER stress-induced cell killing has been linked to the actions of caspase-2, caspase-4, and caspase-12 (39). Caspase-2, caspase-4, and caspase-12, in a cell type-dependent manner, have also been linked to BID cleavage/mitochondrial dysfunction and to activation of caspase-9 and caspase-3. As PERK played a role in MDA-7/IL-24 toxicity, we investigated whether caspase-2 and caspase-4 represent additional proapoptotic signals and noted that knockdown of both caspase-2 and caspase-4 expression suppressed GST-MDA-7 lethality. The activation of caspase-2 and caspase-4 was also secondary to JNK signaling, arguing that the activation of these proteins represent a relatively late event compared with PERK-JNK pathway activation.

¹⁰Unpublished observation.

Based on our findings, other ERK1/2-regulated BH3 domain proteins are also potential targets of MDA-7/IL-24: BAD and BIM are both phosphorylated and inactivated by ERK1/2 phosphorylation and both proteins were dephosphorylated together with ERK1/2 by higher GST-MDA-7 concentrations, suggesting a role in GST-MDA-7 lethality. In transformed fibroblasts, loss of BAK function also modestly reduced GST-MDA-7 lethality. Consequently, our data strongly argue that at least four, possibly five, BH3 domain-containing proteins potentially mediate GST-MDA-7 toxicity downstream of GST-MDA-7-stimulated activation of PERK and JNK1-3 in addition to PERK-stimulated inactivation of ERK1/2. Based on these findings, it is tempting to speculate that the reason why multiple transformed cell types exhibit MDA-7/IL-24 toxicity regardless of genetic background is due to the pleiotropic range of proapoptotic BH3 domain-containing proteins that can be recruited by this cytokine to initiate cell death processes at the level of the mitochondrion.

As GST-MDA-7 causes cell killing in part via a PERK-and cathepsin B-dependent mechanism, that PERK is a sensor of ER stress, and that MDA-7/IL-24 has been shown previously to bind to a regulatory chaperone of PERK, that is, BiP/GRP78, we explored whether GST-MDA-7 altered intracellular vacuolization of cells and specifically whether GST-MDA-7 could cause the formation of autophagic vesicles. Using a plasmid expressing a GFP-tagged form of LC3, GST-MDA-7 caused vacuolization of LC3-GFP in multiple GBM cell types within 12 to 24 h, at a time before measurable cell killing. Expression of a dnPERK protein, knockdown of ATG5 or Beclin-1 protein expression, or overexpression of the MDA-7/IL-24 binding partner BiP/ GRP78 suppressed vesicle formation and protected GBM cells from GST-MDA-7 toxicity (30–33). 3MA can suppress autophagic vesicle formation, and incubation of GBM cells with this agent also suppressed LC3-GFP-containing vesicle formation and protected cells from GST-MDA-7 toxicity. Our data strongly argue that GST-MDA-7 promotes GBM and transformed cell death and one of the earliest manifestations of GST-MDA-7-induced cellular dysfunction is the formation of autophagic vesicles.

Increased expression of HSP70 has been shown by several groups to stabilize endosomes, to suppress the apoptotic activity of apoptosis-inducing factor, and collectively to promote cell survival in response to noxious stresses, including ER stress (44–49). In our analyses, we showed that GST-MDA-7 variably caused early, and definitively caused later, suppression of HSP70 protein levels that correlated with increasing amounts of autophagic vacuolization in glioma cells; overexpression of HSP70 blocked the formation of GFP-LC3 vacuoles and significantly suppressed GST-MDA-7 toxicity. Many laboratories are attempting to generate small-molecule HSP70 inhibitors and it will be of interest to determine whether MDA-7 lethality will be enhanced by any such putative HSP70 inhibitory drug.

In summary, in transformed cells, GST-MDA-7 induces multiple proapoptotic pathways to promote cell death. In primary human GBM cells, activation of the JNK1-3 pathway represents a key nodal signal, downstream of PERK in promoting the activation of multiple proapoptotic proteases and causing mitochondrial dysfunction. Further studies are necessary to define the precise role of PERK in JNK1-3 activation in this cell type. From our studies, it is clear that the downstream effectors are complex, but the defining events in MDA-7/IL-24 promoted lethality of GBM cells involve a shift in the balance between antiapoptotic and proapoptotic signals, eliciting mitochondrial dysfunction uniquely in the context of cancer cells. Defining the critical intracellular signaling events that are relevant in MDA-7/IL-24 cancer-selective lethality *in vitro*, and ultimately in patients, will represent an entry point to develop rationally designed combinatorial approaches with enhanced therapeutic efficacy in malignant glioma and other cancers (9–15).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

GST-MDA-7 causes a caspase-9-dependent induction of primary human glioma cell death. **A**, primary human glioma cells (GBM6) were treated 24 h after plating with GST-MDA-7 (30 nmol/L). At the indicated time points after GST-MDA-7 treatment, GBM6 cells were isolated and subjected to SDS-PAGE, and immunoblotting was done to measure the release of cytochrome *c* into the cytosol, the expression of cleaved caspase-3 and β-actin, and cell killing by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and cell killing by Hoechst assay ($n = 2$). **B**, *top*, GBM6 cells were treated with 1 or 30 nmol/L GST-MDA-7/GST 36 h after plating. Cells were isolated 48 h after exposure and 100 μg protein was subjected to SDS-PAGE on 12% gels followed by immunoblotting to determine the phosphorylation status of ERK1/2 and JNK1-3 and total expression of ERK2 ($n = 4$). *Bottom*, GBM6 cells were cultured for 24 h and then treated with the JNK inhibitory peptide based on sequence from JIP-1 (10 μmol/L; *JNK-IP*) 30 min before addition of GST

or GST-MDA-7 (30 nmol/L). Cells were isolated 24 h after GST-MDA-7 treatment and cell lysates were split into two portions. In one portion, equal amounts of protein lysate were subjected to immunoprecipitation using the anti-active BAX 6A7 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted for BAX. In the second portion, equal amounts of protein lysate were subjected to SDS-PAGE and immunoblotted for phosphorylated JNK1-3 (*P-JNK1-3*), BCL-x_L, cleaved caspase-3, BAX, BAD, BAD S112, BIM, and ERK2. Data are from a representative experiment (*n* = 3). **C**, GBM6 cells were plated and 12 h after plating infected at a multiplicity of infection of 50 to express no gene (*CMV*), dominant-negative caspase-9 (*dncasp9*), or treatment with the JNK inhibitory peptide (10 μmol/L) as indicated. Twenty-four hours after infection, cells were treated with 30 nmol/L GST or GST-MDA-7, and 72 h after GST-MDA-7 treatment, cells were isolated and cell viability was determined by using Annexin V/PI staining assays in triplicate using a flow cytometer (±SE; *n* = 5; #, *P* < 0.05, lower amount of cell killing than vehicle-treated cells). *Top inset*, at 48 h after 30 nmol/L GST-MDA-7 or GST treatment, GBM6 cells expressing dominant-negative caspase-9 or the caspase-8 inhibitor CRM A were isolated and subjected to SDS-PAGE and immunoblotting was done to measure the expression of ERK2 and the levels of cleaved caspase-3 (*n* = 2). **D**, SV40 large T protein transformed MEFs, WT, lacking BIM (*BIM*^{-/-}), lacking BAK (*BAK*^{-/-}), lacking BAX (*BAX*^{-/-}), lacking BAK and BAX (*BAK*^{-/-}*BAX*^{-/-}), and lacking BID (*BID*^{-/-}) were plated and 24 h after plating were treated with 100 nmol/L GST-MDA-7 or GST. Seventy-two hours after GST-MDA-7 treatment, cells were isolated and cell viability was determined by trypan blue exclusion assays in triplicate using a hemacytometer. Data are presented as the true percentage increase in cell death above GST-treated cells (±SE; *n* = 5; #, *P* < 0.05, lower amount of cell killing than WT cells). *Inset*, MEF cells were plated and 24 h after plating treated with 100 nmol/L GST-MDA-7 (*M7*) or GST. Twenty-four and 48 h after GST-MDA-7 treatment, cells were isolated, the cytosol was isolated and subjected to SDS-PAGE, and immunoblotting was done to measure the expression of cytochrome *c* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; *n* = 3).

Figure 2. Cathepsin B – dependent cleavage of BID plays an important role in GST-MDA-7 toxicity in transformed cells. **A**, mouse immortalized embryonic fibroblasts (WT; cathepsin B^{-/-}) were cultured for 36 h and then treated with GST or GST-MDA-7 (0–100 nmol/L, as indicated). Cells were isolated for viability analyses 72 h after GST-MDA-7 treatment as judged in triplicate by trypan blue dye exclusion assay ($n = 3$). *Top flow cytometry*, WT and cathepsin B^{-/-} cells were cultured for 36 h and then treated with GST or GST-MDA-7 (150 nmol/L). Cells were isolated for Annexin V/PI viability analyses 72 h after GST-MDA-7 treatment as judged in triplicate ($n = 3$). *Top immunoblotting*, 48 hours after GST-MDA-7 treatment, WT and cathepsin B^{-/-} cells were isolated, the cytosol fraction was isolated and subjected to SDS-PAGE, and immunoblotting was done to measure the levels of cytochrome *c* and GAPDH ($n = 3$). **B**, *top (i)*, MEF cells were isolated for immunoblotting 48 h after GST-MDA-7 treatment, as per **A**, and 100 μ g protein at each time point was subjected to SDS-PAGE on 12% gels followed by immunoblotting to determine the total expression of ERK2, pro-caspase-3, and BID (representative blots; $n = 3$); *Bottom (ii)*, left, GBM6 cells were cultured for 24 h after plating and then treated with the cathepsin B inhibitor (1 μ mol/L; *cath. B inhib.*) 30 min before addition of GST or GST-MDA-7 (30 nmol/L). Cells were

isolated 48 h after GST-MDA-7 treatment and 100 µg protein at each time point was subjected to SDS-PAGE on 12% gels followed by immunoblotting to determine the total expression of ERK2, pro-caspase-3, and BID (representative blots; $n = 3$). *Right*, GBM6 cells were plated and cultured for 36 h and then treated with the JNK inhibitory peptide (10 µmol/L) 30 min before addition of GST or GST-MDA-7 (30 nmol/L). Cells were isolated 24 h after GST-MDA-7 treatment and equal amounts of protein lysate were subjected to SDS-PAGE and immunoblotted for expression of p43 cathepsin B and ERK2. Data are from a representative experiment ($n = 3$).

Figure 3. GST-MDA-7 promotes transformed cell killing through a PERK-dependent pathway. **A**, transformed MEFs: WT or lacking expression of PERK (PERK^{-/-}) were cultured for 36 h and then treated with GST or GST-MDA-7 (0–60 nmol/L, as indicated). Cells were isolated for viability analyses 72 h after GST-MDA-7 treatment as judged in triplicate by trypan blue dye exclusion assay ($\pm$ SE; $n = 3$; *, $P < 0.05$, greater than WT cells). **B**, WT and PERK^{-/-} cells were cultured for 36 h and then treated with GST or GST-MDA-7 (100 nmol/L). Cells were isolated for Annexin V/PI viability analyses 72 h after GST-MDA-7 treatment as judged in triplicate ($n = 3$). *Top immunoblotting*, 48 h after GST-MDA-7 and JNK inhibitory peptide treatment, WT and PERK B^{-/-} cells were isolated, the cytosol was isolated and subjected to SDS-PAGE, and immunoblotting was done to measure the expression of cytochrome *c* and GAPDH ($n = 3$). **C**, *top (i)*, MEFs as indicated (WT, cathepsin B^{-/-}, and PERK^{-/-}) were treated with GST or GST-MDA-7 (60 nmol/L) and 48 h after treatment isolated. Equal amounts of protein lysate were subjected to SDS-PAGE and immunoblotted for expression of p43 cathepsin B, BID, and ERK2 and for phosphorylation of JNK1/2 (P-JNK1/2). Data are from a representative experiment ($n = 3$). *Bottom (ii)*, WT and PERK^{-/-} fibroblasts were treated with GST or GST-MDA-7 (60 nmol/L) and 48 h after treatment isolated. Equal amounts of protein lysate were subjected to SDS-PAGE and immunoblotted for expression of BCL-x_L, ERK2, and eIF2 α and for phosphorylation of ERK1/2 and eIF2 α (S51). Data are from a representative experiment ($n = 2$). **D**, GBM6 cells were plated and 12

h after plating portions of these cells were transfected with a scrambled siRNA (*SCR*), a siRNA to knockdown caspase-2 expression (*si-2*), or a siRNA to knockdown caspase-4 expression (*si-4*). Other portions of cells were not transfected but treated with either vehicle (DMSO; *VEH*) or with the JNK inhibitory peptide (10 μ mol/L). Forty-eight hours after transfection, cells were treated with GST or GST-MDA-7 at the indicated concentrations. Forty-eight hours (immunoblotting inset to the *right*) or, for viability analyses, 72 h after GST-MDA-7 treatment, cells were isolated as judged in triplicate by trypan blue dye exclusion assay ($\pm$ SE; $n = 2$; #, $P < 0.05$, less than scrambled siRNA – transfected or vehicle-treated cells treated with the same concentration of GST-MDA-7).

Figure 4. GST-MDA-7 causes vacuolization in transformed fibroblasts in a PERK-dependent and eIF2 α -independent manner. **A**, (i), left, transformed MEFs (WT; deleted for PERK, PERK $-/-$, expressing dominant-negative eIF2 α S51A, eIF2 α S51A) were plated in four-well glass chamber slides in triplicate and 24 h after plating treated with GST or GST-MDA-7 (100 nmol/L). Twenty-four hours after GST-MDA-7 treatment, Lysotracker Red was added to the

culture medium and the cells were examined under visual light (visible) or under fluorescent light (Lysotracker Red) at $\times 40$ magnification. Representative images from the triplicate plating ($n = 2$). **(ii)**, *right*, WT MEF and U251 and GBM6 human glioma cells were treated with GST or GST-MDA-7 (100 nmol/L) and coincubated for the 24 h GST-MDA-7 treatment with vehicle (PBS) or with 3MA (5 mmol/L). Twenty-four hours after GST-MDA-7 treatment, Lysotracker Red was added to the culture medium and the cells were examined under visual light (visible) or under fluorescent light (Lysotracker Red) at $\times 40$ magnification. Representative images from the triplicate plating ($n = 2$). **B**, U251 cells were plated in four-well chamber glass slides in triplicate and 12 h after plating were transfected with a plasmid to express LC3-GFP. Twelve hours after transfection, cells were pretreated with vehicle (PBS) or with 3MA (5 mmol/L) and 30 min later were treated with GST or GST-MDA-7 (100 nmol/L). Twenty-four hours after GST-MDA-7 exposure, the U251 cells were examined under visual light (visible) or under fluorescent light (LC3-GFP) at $\times 40$ magnification. Representative images from the triplicate plating ($n = 2$). **C**, **(i)**, GBM6 and U251 cells were plated in four-well chamber glass slides in triplicate and 12 h after plating transfected with a plasmid to express LC3-GFP and in parallel cotransfected with either a vector control plasmid (CMV) or with a plasmid to express dnPERK. Twelve hours after transfection, cells were treated with GST or GST-MDA-7 (100 nmol/L). Twenty-four hours after GST-MDA-7 exposure, the GBM6 and U251 cells were examined under visual light (visible) or under fluorescent light (LC3-GFP). Representative images from the triplicate plating ($n = 2$). **(ii)**, U251 cells were plated in four-well chamber glass slides in triplicate and 12 h after plating transfected with a plasmid to express LC3-GFP and in parallel cotransfected with either a vector control plasmid to express a nonspecific scrambled siRNA (*siSCR*) or plasmids to knockdown expression of Beclin-1 (*siBeclin-1*) or ATG5 (*siATG5*). Parallel studies also transfected cells with plasmids to express scrambled siRNA and untagged GFP. Twelve hours after transfection, cells were treated with GST or GST-MDA-7 (100 nmol/L). Twenty-four hours after GST-MDA-7 exposure, the U251 cells were examined under fluorescent light (LC3-GFP and GFP). Representative images from the triplicate plating ($n = 3$). **Immunoblotting**, cells transfected with siRNA constructs to modulate the expression of ATG5 and Beclin-1 were immunoblotted to determine the expression of Beclin-1 and ATG5 48 h after transfection. Cells treated with GST-MDA-7 and GST were immunoblotted 48 h after treatment to determine the expression of Beclin-1, ATG5, the cleavage status of LC3 and GAPDH ($n = 2$). **D**, transformed MEFs (WT; deleted for PERK, PERK $^{-/-}$) 24 h after plating were treated with GST or GST-MDA-7 (100 nmol/L). Twenty-four hours after GST-MDA-7 treatment, cells were isolated and subjected to SDS-PAGE to determine the expression of Beclin-1, ATG5, the cleavage status of LC3 and GAPDH ($n = 2$).

Figure 5.

GST-MDA-7 causes LC3-GFP vacuolization that is blocked by overexpression of BiP/GRP78. **A, bottom**, U251 and GBM6 cells were plated and 24 h after plating were treated with vehicle (PBS, vehicle) or with 3MA (5 mmol/L), followed 30 min later by treatment with either GST or GST-MDA-7 (30 nmol/L). Forty-eight hours after GST-MDA-7 treatment cells were isolated for viability analyses as judged in triplicate by trypan blue dye exclusion assay ($\pm$ SE; $n = 3$; #, $P < 0.05$, less than amount of death induced by GST-MDA-7 in absence of 3MA). **Top inset**, GBM6 cells were plated in four-well chamber glass slides in triplicate and 12 h after plating were transfected with a plasmid to express LC3-GFP and in parallel cotransfected with either a vector control plasmid (CMV) or with a plasmid to express BiP/GRP78. After transfection (12 h), cells were treated with GST or GST-MDA-7 (100 nmol/L). After GST-MDA-7 exposure (24 h), the GBM6 cells were examined under

visual light (visible) or under fluorescent light (LC3-GFP). The mean $\pm$ SE number of LC3-GFP vesicles per cell ($n = 40$) is indicated. Representative images from a triplicate plating ($n = 2$). **B**, GBM6 and U251 cells were plated in four-well chamber glass slides in triplicate and 12 h after plating transfected with either a vector control plasmid to express a nonspecific scrambled siRNA or plasmids to knockdown expression of Beclin-1 or of ATG5. Twelve hours after transfection, cells were treated with GST or GST-MDA-7 (30 nmol/L). Forty-eight hours after GST-MDA-7 exposure, the viability of the GBM6 and U251 cells was determined by terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling assay on isolated cells ($\pm$ SE; $n = 3$). Data shown are for GBM6 (terminal deoxynucleotidyl transferase– mediated dUTP nick end labeling) and U251 cells was determined by Annexin V/ PI flow cytometry ($\pm$ SE; $n = 2$). **C**, U251 and GBM6 cells were treated with GST or with GST-MDA-7 (30 nmol/L). At the indicated time points, cells were isolated and lysates were subjected to immunoblotting to determine the expression of HSP70, BiP/ GRP78, and GAPDH and the phosphorylation of PERK and eIF2 α S51 ($n = 2$). **D**, U251 cells were plated and 12 h after plating infected at a multiplicity of infection of 50 to express no gene or HSP70. Twelve hours after infection, cells were transfected with a plasmid to express LC3-GFP. Twelve hours after transfection, cells were treated with GST or GST-MDA-7 (100 nmol/L). After GST-MDA-7 exposure (24 and 48 h), the U251 cells were examined under visual light (visible) or under fluorescent light (LC3-GFP). The mean $\pm$ SE number of LC3-GFP vesicles per cell ($n = 40$) is indicated (*, $P < 0.05$, greater than corresponding 24-h value; #, $P < 0.05$, less than value in CMV-infected cells). Representative images from a triplicate plating ($n = 2$).

Figure 6.

GST-MDA-7 causes cell killing by promoting PERK-dependent vacuolization and JNK pathway activation in transformed cells. **A**, U251 cells were plated and 12 h after plating infected at a multiplicity of infection of 50 to express no gene or HSP70. Twenty-four hours after infection, cells were treated with GST or GST-MDA-7 (100 nmol/L). Forty-eight hours after GST-MDA-7 exposure, the viability of the U251 cells was determined by trypan blue exclusion assay and by Annexin V/PI staining ($\pm$ SE; $n = 2$; #, $P < 0.05$, less than value in CMV-infected cells). **B**, treatment of transformed cells with GST-MDA-7 causes PERK-dependent activation of the JNK pathway and PERK-dependent inactivation of the ERK1/2 pathway. JNK pathway signaling plays a key role in the activation of caspase-2 and caspase-4 and cathepsin B; cathepsin B promotes the activation of the BH3 domain protein BID. JNK pathway signaling also activates BAX. Inactivation of ERK1/2 promotes activation of BAD and BIM. Thus, GST-MDA-7, via PERK signaling, promotes activation of at least four BH3 domain proteins, which cause mitochondrial dysfunction and promote cell killing.

GST-MDA-7 causes a caspase-9-dependent induction of primary human glioma cell death

Table 1

Treatment: caspase inhibitor	GBM5 GST	GBM5 GST-MDA-7	GBM6 GST	GBM6 GST-MDA-7	GBM12 GST	GBM12 GST-MDA-7
Vehicle	2.2 ± 0.6	34.0 ± 2.1	4.4 ± 0.6	45.2 ± 2.3	2.1 ± 0.8	20.3 ± 0.8
z-VAD	2.9 ± 1.0	7.7 ± 0.6*	6.4 ± 0.4	11.7 ± 1.5*	4.0 ± 1.2	9.1 ± 1.5*
IETD	3.4 ± 1.0	34.4 ± 2.9	4.8 ± 0.2	45.0 ± 7.2	3.5 ± 1.4	20.7 ± 2.7
LEHD	3.6 ± 7.5	8.9 ± 1.7*	3.3 ± 2.3	12.0 ± 1.5*	4.7 ± 1.8	11.0 ± 0.6*

NOTE: Primary human glioma cells (GBM5, GBM6, and GBM12) were treated 24 h after plating with vehicle (DMSO), a pan-caspase inhibitor (z-VAD), a caspase-8 specific inhibitor (IETD), or a caspase-9 specific inhibitor (LEHD; all at 50 μmol/L) followed 30 min later by treatment of cells with 20 nmol/L GST-MDA-7 or GST. Cell viability was determined 96 h after GST-MDA-7 treatment by trypan blue exclusion assays in triplicate using a hemacytometer (±SE; n = 5).

* P < 0.05, lower amount of cell killing than vehicle-treated cells.

Table 2
Cathepsin B plays an important role in GST-MDA-7 toxicity in transformed cells

Cell type	Treatment					
	Vehicle	z-VAD	LEHD	CMV vehicle	Dominant-negative caspase-9 vehicle	CMV cathepsin B inhibitor
WT MEF + GST	2.1 ± 0.2	2.5 ± 0.2	2.0 ± 0.3			
WT MEF + GST-MDA-7	17.4 ± 0.3	6.3 ± 0.6*	6.9 ± 0.4*			
Cathepsin B ^{-/-} MEF + GST	1.6 ± 0.4	2.9 ± 0.7	2.7 ± 0.5			
Cathepsin B ^{-/-} MEF + GST-MDA-7	6.8 ± 0.5*	2.7 ± 0.3*	3.1 ± 0.6*			
GBM6 + GST				3.4 ± 0.3	5.0 ± 0.8	5.7 ± 0.4
GBM6 + GST-MDA-7				24.2 ± 1.2	7.5 ± 0.2*	9.5 ± 1.1*
GBM12 + GST				2.0 ± 0.1	2.5 ± 0.2	3.6 ± 0.5
GBM12 + GST-MDA-7				16.0 ± 0.6	5.3 ± 0.4*	5.4 ± 0.7*

NOTE: MEFs (WT; cathepsin B^{-/-}) were plated and 36 h after plating were pretreated with vehicle (DMSO), pan-caspase inhibitor (z-VAD, 50 μmol/L), or caspase-9 inhibitor (LEHD, 50 μmol/L). Thirty minutes after inhibitor addition, cells were treated with GST or GST-MDA-7 (60 nmol/L). Inhibitors were resupplemented every 24 h. Cells were incubated for 72 h after which time cell viability was assessed in triplicate by trypan blue assay (±SE; n = 3). In parallel, primary human glioma cells (GBM6 and GBM12) were plated and 12 h after plating were infected with adenoviruses at a final multiplicity of infection of 50 to express as per Fig. 1: CMV or dominant-negative caspase-9. Cells were cultured for 24 h after infection and then treated with the cathepsin B inhibitor (1 μmol/L, cathepsin B inhibitor) 30 min before addition of GST or GST-MDA-7 (30 nmol/L). Cells were incubated for 72 h after which time cell viability was assessed in triplicate by trypan blue assay (±SE; n = 3; #, P < 0.05, lower than CMV-infected vehicle-treated cells with GST-MDA-7 exposure; ##, P > 0.05, no difference between GST- and GST-MDA-7-treated cells).

* P < 0.05, lower than vehicle-treated cells with GST-MDA-7 exposure.

Table 3

dnPERK or 3MA treatment block LC3-GFP vesicle formation by GST-MDA-7

CMV + vehicle + GST	CMV + vehicle + GST - MDA-7	dnPERK + vehicle + GST	dnPERK + vehicle + GST + MDA-7	CMV + 3MA + GST	CMV + 3MA + GST-MDA-7
1.6 ± 0.5	5.1 ± 0.8 *	1.3 ± 0.4	1.5 ± 0.3 †	1.1 ± 0.4	1.9 ± 0.4 †

NOTE: U251 cells were plated in four-well chamber glass slides in triplicate and 12 h after plating were transfected with a plasmid to express LC3-GFP and in parallel cotransfected with either a vector control plasmid (CMV) or with a plasmid to express dnPERK. Twelve hours after transfection, cells were pretreated with vehicle (PBS) or 3MA (5 mmol/L) and 30 min later were treated with GST or GST-MDA-7 (100 nmol/L). Twenty-four hours after GST-MDA-7 exposure, the U251 cells were examined under visual light or under fluorescent light at ×40 magnification. The mean number of intense LC3-GFP staining vesicles per cell was determined from multiple fields from multiple treatment conditions (±SE; n = 40 cells; n = 3 separate experiments; a representative is shown).

* P < 0.05 greater than value in CNV + Vehicle + GST.

† P < 0.05 less than value in CNV + Vehicle + GST - MDA-7.

Table 4

Knockdown of Beclin-1 or ATG5 expression blocks LC3-GFP vesicle formation by GST-MDA-7

siSCR + GST	siSCR + GST-MDA-7	siATG5 + GST	siATG5 + GST + MDA-7	siBeclin-1 + GST	siBeclin-1 + GST-MDA-7
1.4 ± 0.42	5.4 ± 0.64 [*]	0.7 ± 0.2	1.3 ± 0.4 [†]	0.9 ± 0.4	1.2 ± 0.3 [†]

NOTE: U251 cells were plated in four-well chamber glass slides in triplicate and 12 h after plating were transfected with a plasmid to express LC3-GFP and in parallel cotransfected with either a vector control scrambled siRNA plasmid (siSCR) or plasmids to knockdown the expression of either Beclin-1 (siBeclin-1) or ATG5 (siATG5). Twelve hours after transfection, cells were treated with GST or GST-MDA-7 (100 nmol/L). Twenty-four hours after GST-MDA-7 exposure, the U251 cells were examined under visual light or under fluorescent light at ×40 magnification. The mean number of intense LC3-GFP staining vesicles per cell was determined from multiple fields from multiple treatment conditions (±SE; *n* = 40).

^{*} *P* < 0.05 greater than value in CNV + Vehicle + GST.

[†] *P* < 0.05 less than value in CNV + Vehicle + GST – MDA-7.