MDA-7/IL-24 as a cancer therapeutic: from bench to bedside

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The novel cytokine melanoma differentiation associated gene-7 (*mda*-7) was identified by subtractive hybridization in the mid-1990s as a protein whose expression increased during the induction of terminal differentiation, and that was either not expressed or was present at low levels in tumor cells compared with non-transformed cells. On the basis of conserved structure, chromosomal location and cytokine-like properties, MDA-7, has now been classified as a member of the expanding interleukin (IL)-10 gene family and designated as MDA-7/IL-24. Multiple studies have shown that the expression of MDA-7/IL-24 in a wide variety of tumor cell types, but not in the corresponding equivalent non-transformed cells, causes their growth arrest and ultimately cell death. In addition, MDA-7/IL-24 has been noted to be a radiosensitizing cytokine, which is partly because of the generation of reactive oxygen species and ceramide that cause endoplasmic reticulum stress. Phase I clinical trial data has shown that a recombinant adenovirus expressing MDA-7/IL-24 [Ad.*mda*-7 (INGN-241)] was safe and had measurable tumoricidal effects in over 40% of patients, which strongly argues that MDA-7/IL-24 may have significant therapeutic value. This review describes what is known about the impact of MDA-7/IL-24 on tumor cell biology and its potential therapeutic applications. *Anti-Cancer Drugs* 21:725–731 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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The discovery of MDA-7/IL-24 and its biologic effects

MDA-7/IL-24 was discovered using a subtraction hybridization approach by exposing melanoma cells to the terminal differentiation-inducing agents interferon β and mezerein [1–3]. On the basis of a conserved amino acid signature sequence, chromosomal location and cytokine-like properties, *mda*-7, has been classified as a member of the expanding interleukin (IL)-10 gene family, which includes IL-10, IL-19, IL-20, IL-22 and IL-26, and has been designated as MDA-7/IL-24 [3–8]. MDA-7/IL-24 protein expression is decreased in advanced melanomas, with nearly undetectable levels in metastatic disease in general agreement with this gene product being classified as a tumor suppressor [3–5]. Other published studies over the last 15 years have shown that enforced expression of MDA-7/IL-24, either by transfection of tumor cells with a plasmid containing the cDNA for MDA-7/IL-24 or by use of a recombinant adenovirus to deliver the gene, Ad.*mda*-7, rapidly inhibits the growth of a broad-spectrum of cancer cells, resulting in tumor cell death within 24–48 h [1–11]. When expressed, MDA-7/IL-24 is secreted from cells, as would be expected for a cytokine. Of considerable note, when MDA-7/IL-24 was overexpressed in non-transformed cells, little change was observed in either cell growth or cell viability [1–12].

Initial studies using the mammalian cell synthesized MDA-7/IL-24 protein, a protein that is a dimer and glycosylated, showed that purified MDA-7/IL-24 interacted with two type II cytokine hetero-dimeric receptor complexes: IL-20R1/IL-20R2 (type 1 IL-20R) and IL-22R1/IL-20R2 (type 2 IL-20R) (Fig. 1) [13]. In one of the first of these studies, non-transformed BHK cells stably transfected with IL-20 and IL-22 receptors were treated with MDA-7/IL-24; at low pmol/l concentrations of MDA-7/IL-24 (< 100 pmol/l) growth was promoted, whereas at higher concentrations (> 100 pmol/l) it inhibited cell proliferation. In cells transfected to express IL-20 receptor complexes, MDA-7/IL-24 activated multiple STAT transcription factors. However, in ovarian carcinoma cells, which express endogenous IL-20 receptor complexes, it was noted that MDA-7/IL-24 at low nmol/l concentrations promoted growth inhibition

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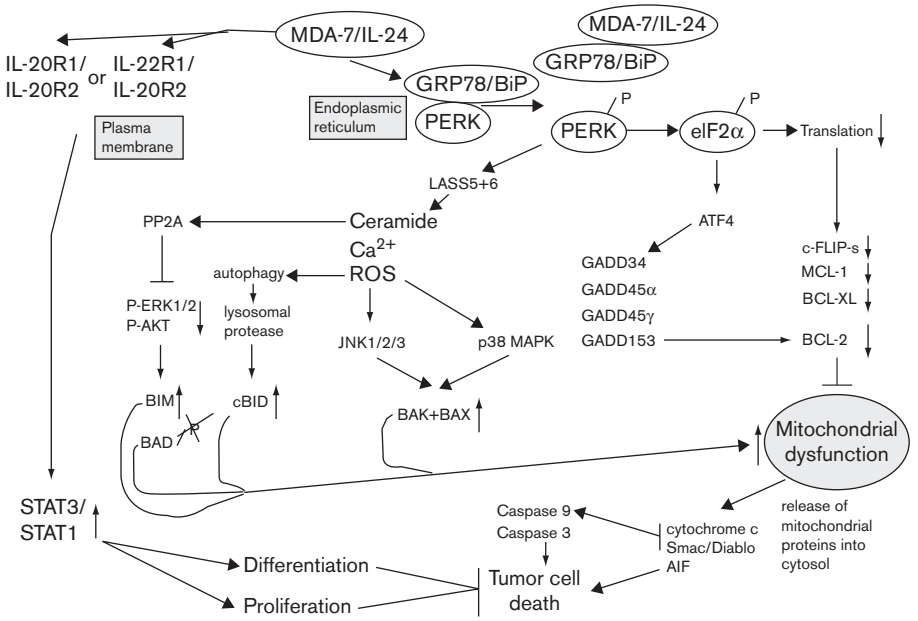
Fig. 1



Molecular pathways by which MDA-7/ interleukin (IL)-24 regulates cell viability and cell growth. MDA/IL-24 has two major targets in cells: the IL-20/IL-22 receptor complexes and the HSP70 family chaperone GRP78/BiP. MDA-7/IL-24 binding to its cognate receptors activates the STAT family transcription factors and activation of these factors can promote differentiation and proliferation in a cell type-dependent manner. STAT transcription factors play no role in MDA-7/IL-24 toxicity. MDA-7/IL-24 binds to GRP78/BiP; it is possible that entry of the bacterial synthesized GST-MDA-7 into the tumor cells is mediated by binding to cell surface GRP78/BiP. The majority of GRP78/BiP is present in the endoplasmic reticulum and is bound to PKR-like endoplasmic reticulum kinase (PERK); the chaperone inhibits PERK kinase activity. MDA-7/IL-24 disrupts the association of GRP78/BiP with PERK permitting PERK to phosphorylate eIF2α; phospho-eIF2α suppresses the translation of the majority of cellular proteins resulting in the rapid loss of protective proteins that have short half-lives such as MCL-1 and BCL-XL, and through ATF4, promotes the transcription of a specific subset of genes example, GADD34, that promote apoptosis. PERK signaling promotes increased LASS6 (ceramide synthase 6) levels that promote increased Ca^{2+} mobilization leading to elevated reactive oxygen species (ROS) levels. Increased ceramide/Ca^{2+}/ROS activate c-Jun NH2-terminal kinase (JNK) and p38 signaling that promotes activation of the toxic BH3 domain proteins BAX and BAK.

without altering the STAT transcription factor phosphorylation/function [13,14]. Other studies have shown using tumor cells that lack STAT1 or STAT3 function or with blocked Janus kinase function that STAT pathway signaling is not required for MDA-7/IL-24-induced growth arrest or tumor cell killing [15]. These studies used both the viral and protein delivery of MDA-7/IL-24 to show a lack of STAT factor involvement.

More recently, studies have indicated a difference in the cell signaling and cell killing properties between bacterial synthesized unglycosylated and monomeric GST-MDA-7/IL-24 and mammalian cell synthesized glycosylated dimeric MDA-7/IL-24 with FLAG or $(His)_6$ tags to aid its purification. In multiple studies using a wide variety of transformed cell lines, GST-MDA-7/IL-24 has been noted to promote cell growth arrest and apoptosis in a tumor cell-specific manner and has been noted to cause these effects independent of the expression of IL-20 receptor complexes, in a similar manner to the intracellular delivery of MDA-7/IL-24 through infection with Ad.*mda*-7 ([16,17], and references therein). This would suggest that GST-MDA-7/IL-24 is taken up by cancer cells in an IL receptor-independent manner. In contrast to GST-MDA-7/IL-24 and Ad.*mda*-7, purified MDA-7/IL-24, synthesized in mammalian cells, does not seem to have any biologic effect on the cells lacking expression of IL-20 receptor complexes. Of note, however, in the cells in which the IL-20 receptor complexes were expressed, mammalian synthesized MDA-7/IL-24-induced cell killing was independent of STAT transcription factor activation, in a similar manner to GST-MDA-7/IL-24 and Ad.*mda*-7. For example, in A549 human lung carcinoma cells, which lack expression of the IL-20 receptor complexes, extracellular treatment with mammalian cell synthesized MDA-7/IL-24 results in no biologic effect on cell growth/viability. In contrast, treatment with GST-MDA-7/IL-24, or viral infection with Ad.*mda*-7 or Ad.*mda*-7 signal peptide null (SP), which expresses a nonsecreted form of MDA-7/IL-24, or transfection of these cells with a plasmid to express MDA-7/IL-24, results in tumor cell growth arrest and cell death [18,19]. Furthermore, while it has been noted that MDA-7/IL-24, IL-20, and IL-19 activated the STAT transcription factors in the IL-20 receptor expressing cancer cells, only MDA-7/IL-24 has the ability to cause cell death [20]. Thus, the biologic differences between GST-MDA-7/IL-24 and His6-MDA-7/IL-24 and

adenoviral/plasmid delivery of MDA-7/IL-24 into a cell, particularly with respect to findings from the A549 model, is of primary importance in understanding the difference between MDA-7/IL-24 acting as a cytokine in the 'classic' sense of IL biology and MDA-7/IL-24 acting as a protein that causes a toxic endoplasmic reticulum (ER) stress response (see below). It has been the comparison between whether MDA-7/IL-24 was delivered internally or externally in a cell lacking the receptors for the cytokine that ultimately permitted the insight into how MDA-7/IL-24 entered cells and the separation of receptor-mediated signaling from ER stress signaling, and developed a greater understanding of MDA-7/IL-24 as a secreted cytokine with a 'toxic bystander effect'.

MDA-7/IL-24 and transformed cell killing

The pathways by which Ad.*mda*-7 (*or* transfection with a cDNA to express MDA-7/IL-24; treatment with bacterial synthesized GST-MDA-7/IL-24 or eukaryotic cell generated $_{His6}$-MDA-7/IL-24) enhances apoptosis in tumor cells are still not completely understood; however, over the last 5 years a large amount of evidence from multiple studies using each of these tools has shown the involvement of proteins important in the regulation of ER stress and mitochondrial integrity [21–26]. Some studies have argued that MDA-7/IL-24 promoted activation of the double-stranded RNA-activated kinase, protein kinase R (PKR), which was correlated with enhanced eIF2α phosphorylation and MDA-7/IL-24-stimulated cell death. In this study, PKR-null fibroblasts were resistant to IL-24-induced apoptosis, although subsequent studies from the same group have argued that PKR does not always play a role in the lethal effects of MDA-7/IL-24 [27,28].

In studies from our laboratories using GST-MDA-7 and Ad.*mda*-7, we noted that MDA-7/IL-24 protein binds to the HSP70 family chaperone BiP/GRP78 (Fig. 1). Binding of MDA-7/IL-24 to BiP/GRP78 inactivates the chaperone function of the protein promoting its dissociation from PKR-like endoplasmic reticulum kinase (PERK) [21]. Overexpression of BiP/GRP78 suppresses MDA-7/IL-24-induced toxicity [22,23]. Dissociation of BiP/GRP78 from PERK promotes PERK trans-phosphorylation and activation, and subsequently the phosphorylation and activation of eIF2α (Fig. 1). The phosphorylation of eIF2α in turn leads to the global suppression of protein translation, which, with respect to its tumor cell killing properties, results in the reduced expression of anti-apoptotic proteins that have short half-lives such as MCL-1, BCL-XL and c-FLIP-s [29–31]. Indeed, some of the earliest correlative observations regarding MDA-7/IL-24 toxicity were that the cytokine decreased expression of BCL-XL and enhanced expression of toxic BH3 domain proteins such as BAX and BAK ([32,33] and references therein). Activation of eIF2α also activates ATF4, which leads to increased levels of Growth Arrest and Differentiation and Death (GADD) transcription factors such as GADD153 (CHOP) and GADD34. Very recent data from our laboratories has also shown a central role for the PERK-dependent generation of the lipid second messenger species ceramide and dihydroceramide in response to MDA-7/IL-24 [23,34,35]. One mechanism by which MDA-7/IL-24 likely increases dihydroceramide levels in a PERK-dependent manner is by increasing the ceramide synthase 6 protein stability (Fig. 1). Elevated ceramide levels facilitate calcium ion-dependent generation of reactive oxygen species that together play a central role in the modulation of signaling pathway function (see below) and mitochondrial integrity.

What is perhaps more unusual with respect to the cancer therapeutic properties of MDA-7/IL-24 compared with the multiple other FDA approved anticancer agents, and in a manner consistent with the phrase 'water always wins,' is that in all tumor and transformed cells tested to date, intracellular delivery of this cytokine protein causes cell death; however, the precise mode of cytokine lethality exhibits subtle differences between tumor cells of different tissue origins [36,37]. One difference between cell types is the degree to which different toxic BH3 domain proteins play as upstream agonists promoting mitochondrial dysfunction. For example, the ability of Ad.*mda*-7 to induce apoptosis in the prostate cancer cell line, DU145, which does not produce BAX, indicates that MDA-7/IL-24 can mediate apoptosis in tumor cells by a BAX-independent pathway [38]. In multiple primary human glioblastoma cells we noted downstream of PERK activation and lysosomal dysfunction that cathepsin B-dependent cleavage of BID played a central role in cytokine-induced mitochondrial dysfunction and lethality [22–24]. In a cell type-dependent manner, MDA-7/IL-24 inactivates the ERK1/2 and activates the c-Jun NH2-terminal kinase (JNK) signaling pathways leading to the dephosphorylation of BAD S112 and BIM, which promotes BAD activation and BIM protein stabilization and activation of BAX and BAK, respectively. In melanoma cell lines, but not in normal melanocytes, infected by Ad.*mda*-7 or treated with GST-MDA-7, it was noted that a significant decrease in both BCL-2 and BCL-XL levels occurred, with a more modest upregulation of BAX and BAK expression [39]. This data supports a hypothesis that Ad.*mda*-7 or GST-MDA-7 enhances the ratio of pro-apoptotic to anti-apoptotic proteins in cancer cells, thereby facilitating the induction of apoptosis.

MDA-7/IL-24 and lysosomal dysregulation

Increased mitochondrial dysfunction caused by MDA-7/IL-24 has been linked to cytokine-induced ER stress. PERK signaling not only suppresses the expression of both MCL-1 and BCL-XL, but also causes activation of the JNK pathway through PERK-dependent increases in reactive oxygen species (ROS)/ceramide levels, which in turn promotes BAX and BAK activation, and this then

promotes mitochon reatidrial dysfunction (Fig. 1) [23,34,40]. In some cell types, notably only ovarian and renal carcinoma cells at present, MDA-7/IL-24 has been shown to cause activation of the extrinsic apoptosis pathway, in particular the death receptor CD95 [25,26]. In ovarian cancer cells, CD95 activation was ligand independent and required MDA-7/IL-24-induced ceramide generation [25]. Downstream of the CD95 receptor, cleavage of BID again played a central role in mediating cytokine toxicity, though in ovarian and renal carcinoma cells, BID cleavage is caspase 8-dependent rather than cathepsin-dependent as was noted in glioblastoma cells.

MDA-7/IL-24 as a therapeutic tool

As noted earlier, MDA-7/IL-24 is a secreted protein, and secreted MDA-7/IL-24 been shown in several studies to have a '*toxic bystander*' effect on distant tumor cells *in vitro*. From the standpoint of MDA-7/IL-24 as a gene therapeutic tool, based on simple mass action effects, it is not possible to infect every tumor cell within a tumor using an adenovirus even with intra-tumoral injection, and this has been a possible reason why so many gene therapy approaches have failed in the clinic. In addition, systemic IV administration of any recombinant adenovirus will not result in the productive infection of any disseminated tumor because of rapid first pass sequestration of virus by the liver coupled with neutralization by any preexisting anti-adenovirus antibodies. Based on its selective and potent anti-cancer activity *in vitro* and in animal models, a Phase I clinical trial was performed in advanced carcinomas and melanomas using a replication incompetent serotype 5 adenovirus to express MDA-7/IL-24; Ad.*mda*-7 (INGN-241) [17,41–44] (Table 1). These studies used repeated intra-tumoral injection of Ad.*mda*-7 in patients with advanced disease and indicated that repeated administration of Ad.*mda*-7 was safe and this gene could induce apoptosis in a large percentage of tumor volume with a measurable clinical response rate of approximately 44%.

It was evident in this trial that infection of a small proportion of tumor cells with Ad.*mda*-7 resulted in detectable MDA-7/IL-24 protein levels and increased tumor cell apoptosis many centimeters from the site of any virally infected tumor cell in the tumor, indicating, as was observed in animals, and now in patients, that secreted MDA-7/IL-24 was having a '*toxic bystander*' effect on uninfected tumor cells [45–48]. In addition, we have noted in prostate, renal and glioblastoma xenograft tumors that Ad.*mda*-7 infection of an established tumor growing on one flank of an animal results in growth arrest and apoptosis in an uninfected tumor growing on the opposite flank of the animal ([16,48,49]; unpublished results). Clearly, however, at sites more distant to viral administration where MDA-7/IL-24 concentrations may be only growth inhibitory and not cytotoxic, the combination of MDA-7/IL-24 therapy with established therapeutic agents to enhance the toxicity of MDA-7/IL-24 would be of considerable utility. The toxic effects of MDA-7/IL-24 therapy could be combined with other treatment modalities to achieve an improved *profound* clinical response.

MDA-7/IL-24 radiosensitizes tumor cells

MDA-7/IL-24, delivered as either a virus, plasmid, GST-MDA-7/IL-24 or $_{His6}$-MDA-7/IL-24, causes the generation of ROS in tumor cells, but not in non-transformed cells, and quenching of ROS production suppresses MDA-7/IL-24 toxicity. Several long-established therapeutic modalities also generate ROS in tumor cells as part of their toxic biology [50]. For example, ionizing radiation causes ionizing events in water, generating hydroxyl radicals that can impact on the function of mitochondria in cells, which in turn amplify the initial free radical signaling, generating large amounts of reactive oxygen and nitrogen species [51]. In addition, radiation can cause DNA damage, activate poly-ADP ribosyl polymerase leading to an altered cellular redox status because of NADPH depletion, which can also be sensed by the mitochondria [52]. Radiation exposure also increases ceramide levels in tumor cells [53].

Radiotherapy is used as a primary modality for the treatment of many malignancies including those of the breast, brain, prostate, and lung. On the basis of the tumoricidal effects of both radiation and MDA-7/IL-24, it has been a

Table 1 Responses of patients to INGN-241 therapy (cohort 8)

No. of patients	Sex/age	Diagnosis	Previous treatments	No. of injections	Response	Time to death[a]
81	Male/71	Adenocarcinoma	P, T, G	2[b]	SD	nd
83	Female/64	Melanoma	S, RT, IT, IF	24[c]	CR, PR, SD	>600
84	Female/62	Melanoma	S, RT, IT, IF	12	PR	309
85	Male/64	Penile carcinoma	S, RT, CDDP, T	6	PD	75
86	Female/66	NSCLC	RT, P, T	3[b]	SD	180
87	Male/62	SCCHN	S, RT, CDDP, T, F, P	6	PD	185
88	Male/91	Lip carcinoma	S, RT	6	SD	181

Patients in cohort 8 were treated with 2×10^2 virus particles repeated $2 \times$ per week for 3 weeks.
CDDP, cisplatinum; CR, complete response; F, 5-fluorouracil; G, gemcitabine; IF, interferon γ; IT, immuno-therapy; PD, progressive disease; PR, partial response; RT, radiotherapy; S, surgery; SD, stable disease; T, taxane.
[a]Time in days from first injection of INGN-241 until death.
[b]Patient did not complete one full course of treatment (6 injections).
[c]Patient received 12 injections on compassionate use protocol.

logical step for investigators to determine whether MDA-7/IL-24 had radiosensitizing potential. Several laboratories have shown that Ad.*mda*-7, GST-MDA-7/IL-24 and MDA-7/IL-24 can radiosensitize a wide variety of tumor cell lines *in vitro* and *in vivo* [54–58]. In studies using human glioma and prostate carcinoma cells, the ability of both ionizing radiation and MDA-7/IL-24 to generate ROS was directly linked to the radiosensitizing properties of MDA-7/IL-24. MDA-7/IL-24 activates ceramide synthase 6 as part of its toxic effects and ceramide synthase 6 was also linked to MDA-7/IL-24 toxicity; others have shown that radiotherapy uses ceramide synthase 6 to kill tumor cells [59]. Other therapeutic agents have also been shown to act, in part, by generating ROS, including arsenic trioxide, 4-hydroxyphenyl-retinamide (4-HPR), vitamin E and perillyl alcohol [60–66]. In general, agreement with ROS enhancing the lethal actions of MDA-7/IL-24, combined treatment of renal, brain, lung, breast, pancreatic and prostate carcinoma cells with MDA-7/IL-24 and in combination with radiotherapy, arsenic trioxide or 4-hydroxyphenyl-retinamide resulted in a highly synergistic potentiation of tumor cell killing that was not manifested in non-transformed epithelial cell counterparts. Collectively, these findings argue that established and novel therapeutic modalities, which generate ROS, can promote MDA-7/IL-24 lethality in cancer cells.

MDA-7/IL-24 regulates signaling pathways that control the apoptotic threshold and facilitate radiosensitization

The regulation of signal transduction pathway functions by Ad.*mda*-7 and GST-MDA-7/IL-24 protein, particularly when combined with therapeutic modalities that generate ROS and ceramide, is apparently as complicated as the number of mechanisms by which MDA-7/IL-24 has been reported to induce cell death. As noted in an earlier section, activation of the STAT transcription factors does not seem to significantly modulate MDA-7/IL-24 lethality, despite MDA-7/IL-24 activating STAT transcription factors through IL-20 receptor complexes [14,15]. Data in several tumor cell types has argued that either Ad.*mda*-7 or (GST–)MDA-7/IL-24 proteins promotes activation of the p38 mitogen-activated protein kinase (MAPK) pathway, which through GADD34 (CHOP) promotes growth arrest and cell death [54,67]. In part, this may be explained by the data suggesting that MDA-7/IL-24 causes PKR/PERK activation in some tumor cell types, which is a known upstream activator of both p38 MAPK and GADD34. However, in some tumor cell types, MDA-7/IL-24-induced p38 MAPK signaling clearly also plays a 'switch-hitter' role with respect to growth and survival, wherein low concentrations of MDA-7/IL-24 induce a level of p38 MAPK signaling that facilitates growth arrest and cell survival with higher MDA-7/IL-24 concentrations causing an intense sustained pathway activation that leads to tumor cell death [68]. Several studies have linked the JNK pathway as a mediator of MDA-7/IL-24 toxicity; as MDA-7/IL-24 increases ROS and ceramide levels and as these messengers have been widely shown by many groups to strongly activate JNK pathway signaling, this finding is perhaps not too surprising (e.g. [69]). Other studies have shown that MDA-7/IL-24 inhibits PI3K/AKT and ERK1/2 pathway function, which in the case of ERK1/2 signaling is mediated by the MDA-7/IL-24-induced activation of PERK; this reduction in ERK1/2 activity further promotes the MDA-7/IL-24–induced reduction in MCL-1 levels and facilitates JNK pathway activation [68].

As a single agent, ionizing radiation-induced cell killing in a variety of cancer cells has been linked to the activation of the JNK pathway [70,71]. When combined with ionizing radiation, MDA-7/IL-24 has been suggested to promote radiation toxicity by modulating JNK1/2/3 pathway signaling [57,72]. For example, lung cancer cells were radiosensitized by Ad.*mda*-7 through JNK1/2 signaling, without radiation further enhancing MDA-7/IL-24-induced JNK1/2 activation [18,58]. The use of established rodent and human glioma cell lines, and primary human glioma cell isolates, showed that Ad.*mda*-7 caused radiosensitization *in vitro* and *in vivo*, and that in-vitro sensitization was dependent on JNK1/2/3 activation and in-vivo sensitization correlated with increased JNK1/2/3 phosphorylation [57,72,73]. Many groups have argued that prolonged intense JNK1/2/3 pathway signaling is involved in cell death processes.

Conclusion

MDA-7/IL-24 is a multifaceted killer of cancer cells that has shown significant clinical benefit in patients as a single agent. Future clinical studies will be required to determine whether MDA-7/IL-24 represents a viable therapeutic agent in glioblastoma and other cancers, and whether MDA-7/IL-24 can be rationally combined with other established cancer treatments to improve tumor control. On the basis of the remarkable efficacy shown by MDA-7/IL-24 using direct tumor injection in patients with advanced cancers, we are very optimistic that this molecule will display profound activity in patients with diverse cancers, especially when combined with therapeutic agents that promote ER stress responses. We are actively pursuing these combinatorial studies and investigating improved and unique ways of effectively delivering MDA-7/IL-24 *in vivo*. One mechanism to increase the total amount of MDA-7/IL-24 being delivered to the site of the tumor is by the use of a conditionally replicative adenovirus, also termed as cancer terminator viruses. A virus that only replicates in tumor cells will result in viral replication-dependent tumor cell killing and the synthesis and release of MDA-7/IL-24 that will kill and suppress the growth of uninfected tumor cells. On account of the lack of expression of the coxsakie and adenovirus receptor, many tumor cells cannot be

infected by type 5 adenovirus and the development of viruses with chimeric knob proteins to deliver gene therapeutics is also being explored: a type 5/type 3 recombinant adenovirus to deliver MDA-7/IL-24 was recently shown by us to be a more effective therapeutic tool for GBM tumors *in vivo* than a 'standard' type 5 virus [23]. Finally, it is possible that although lethal but highly immunogenic forms of MDA-7/IL-24, such as GST-MDA-7, can be delivered to tumors through their encapsulation in microbubbles, which along with ultrasound will target delivery of this cytokine to cancers [74]. Thus, the possible approaches to deliver MDA-7/IL-24 are diverse in nature, and all the noted approaches will, we hope, be translated into the clinic for evaluation in the next 5 years.

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