Published in final edited form as:

Biochem Biophys Res Commun. 2017 September 16; 491(2): 463–468. doi:10.1016/j.bbrc.2017.07.050.

ML327 Induces Apoptosis and Sensitizes Ewing Sarcoma Cells to TNF-Related Apoptosis-Inducing Ligand

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Abstract

Ewing sarcomas are rare mesenchymal-derived bone and soft tissue tumors in children. Afflicted children with distant metastases have poor survival despite aggressive therapeutics. Epithelial-to-mesenchymal transition in epithelial carcinomas is associated with loss of E-cadherin and resistance to apoptosis. ML327 is a novel small molecule that we have previously shown to reverse epithelial-to-mesenchymal transition features in both epithelial and neural crest-derived cancers. Herein, we sought to evaluate the effects of ML327 on mesenchymal-derived Ewing sarcoma cells, hypothesizing that ML327 initiates growth arrest and sensitizes to TNF-related apoptosis-inducing ligand. ML327 induced protein expression changes, increased E-cadherin and decreased vimentin, consistent with partial induction of mesenchymal-to-epithelial transition in multiple Ewing Sarcoma cell lines (SK-N-MC, TC71, and ES-5838). Induction of epithelial features was associated with apoptosis, as demonstrated by PARP and Caspase 3 cleavage by immunoblotting. Cell cycle analysis validated these findings by marked induction of the subG₀ cell population. *In vitro* combination treatment with TRAIL demonstrated additive induction of apoptotic markers. Taken together, these findings establish a rationale for further *in vivo* trials of ML327 in cells of mesenchymal origin both alone and in combination with TRAIL.

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Conflicts of Interest

The authors declare no potential conflicts of interest.

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Keywords

ML327; Ewing Sarcoma; Epithelial-to-mesenchymal transition; Isoxazole; TRAIL; apoptosis

1. Introduction

Ewing Sarcomas (ES) are rare, aggressive childhood cancers thought to arise from mesenchymal stem cells of bone and soft tissues [1,2,3]. This family of cancers arises due to a balanced chromosomal translocation of the *EWS* gene and a member of the *ETS* family of genes, most commonly friend leukemia virus integration 1 (*FLI1*) [1,4]. Five-year survival rates are 60–70% for children with localized disease, and these rates fall to 30% for children afflicted with metastatic spread [1,4]. Multimodal treatment is the standard of care, encompassing systemic chemotherapy in combination with radiation or surgery [4]. Together, these findings highlight the need to identify novel biologic strategies for the treatment of this disease.

Epithelial-to-mesenchymal transition (EMT) is a dynamic developmental process that is exploited in carcinoma progression to mediate invasion, metastasis, and therapeutic resistance [5,6]. Loss of E-cadherin expression is considered the hallmark event of EMT [7]. We have previously conducted a phenotypic screen of 83,200 compounds to identify small molecules capable of inducing the re-expression of E-cadherin expression in colon and lung carcinoma cell lines [8]. Chemical optimization of our initial “hit” yielded a chemical probe, ML327 (*N*-(3-(2-hydroxynicotinamido) propyl)-5-phenylisoxazole-3-carboxamide), that partially reverses EMT in advanced epithelial cancers [9]. ML327 does not alter the cellular viability of colon and lung cancer cells, but is capable of blocking carcinoma cell invasiveness *in vivo* [10]. Intriguingly, we have recently reported that induction of partial mesenchymal-to-epithelial transition (MET) in neural crest-derived neuroblastomas blocks growth both *in vitro* and *in vivo* by inducing cell cycle arrest and necrosis, highlighting the therapeutic potential of this small molecule in cancers of non-epithelial origin [11].

Lack of progress in the treatment of children with ES has led to investigations into the efficacy of TNF-related apoptosis-inducing ligand (TRAIL) [12,13]. TRAIL is a pro-apoptotic cytokine of the TNF superfamily with appealing therapeutic potential given its ability to selectively induce apoptosis in cancer cells with minimal toxicity [14]. The majority of ES cell lines are sensitive to TRAIL *in vitro* [12]. TRAIL-based strategies have also been shown to block tumor growth and osteolysis and increase survival in *in vivo* ES models [15,16]. Resistance to TRAIL has been linked to acquisition of migratory mesenchymal characteristics and upregulation of anti-apoptotic proteins, including cellular FLICE-like inhibitory protein (cFLIP) [14].

The therapeutic potential of ML327-induced MET against cells of mesenchymal origin has not been explored. In the present study, we hypothesized that induction of MET using ML327 would block the growth of ES cells and sensitize to TRAIL-mediated apoptosis. Herein, we report that ML327 induces apoptosis in ES cells and has additive pro-apoptotic effects when used in combination with TRAIL *in vitro*. These findings provide a rationale to investigate the *in vivo* effects of small molecule-mediated MET agents, such as ML327, in

the treatment of sarcomas, both alone and in combination with TRAIL-based therapeutic strategies.

2. Materials and Methods

2.1. Cell Culture

SK-N-MC cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). TC71 and ES-5838 were kindly provided as a gift from Jialiang Wang, PhD (Vanderbilt University; Nashville, TN). SK-N-MC and TC71 cells both exhibit a *EWS-FLII* translocation, while ES-5838 cells feature an *EWS-ERG* translocation. Cells were maintained in RPMI 1640 with 10% FBS at 37 °C in a humidified atmosphere consisting of 5% CO₂ and 95% air.

2.2. Antibodies and Reagents

E-cadherin antibody was from Cell Signaling Technology (Danvers, MA). Vimentin, Caspase 3 and PARP primary antibodies were obtained from Abcam (Cambridge, MA). cFLIP primary antibody was purchased from Enzo Life Sciences (Farmingdale, NY). TRAIL was purchased from Bio Vision (#4354-50, San Francisco, CA) and was also graciously provided by Dr. Avi Ashkenazi (Genentech, San Francisco, CA). All other reagents were obtained from Sigma (St. Louis, MO).

2.3. Chemical Synthesis

ML327 was synthesized as previously described through the Vanderbilt Institute of Chemical Biology [10]. ML327 was solubilized in DMSO.

2.4. Western Blotting

Whole cell lysates were collected using cell lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, aprotinin, leupeptin, and 1 mM sodium orthovanadate) supplemented with proteinase inhibitors (Roche; Mannheim, Germany) and PMSF (1 mM). Protein (30–70µg) was run on a SDS-PAGE gel, transferred to a PVDF membrane, and probed with antibodies. Blots were developed using an enhanced chemiluminescence substrate (Perkin Elmer; Waltham, MA).

2.5. Cell Cycle Analysis

Cell cycle distribution was analyzed using flow cytometry. 1×10⁶ cells were trypsinized, washed with PBS, and fixed in 70% ethanol. Fixed cells were incubated with RNase (100 µg/mL), stained with propidium iodide (50 µg/mL), and analyzed on a 3-laser BD LSRII (BD Biosciences, San Jose, CA). Flow Cytometry experiments were performed in the VUMC Flow Cytometry Shared Resource.

3. Results

3.1. ML327 Induced Partial MET in ES Cells

We have previously reported partial reversal of TGF-β-induced EMT with ML327 (10 µM) in an immortalized mouse mammary epithelial cell line [10]. This EMT reversal was

associated with upregulation of E-cadherin, a hallmark of epithelial cell fate, as well as a downregulation of the mesenchymal marker, Vimentin. We therefore tested whether ML327 would induce MET features in ES cell lines. We evaluated whether ML327 (10 μ M) was capable of eliciting protein expression changes consistent with MET induction in three ES cell lines (TC71, ES-5838, and SK-N-MC) featuring *EWS*-fusion proteins. Similar to our observations in epithelial and neural crest-derived cell lines, ML327 successfully induced the expression of E-cadherin in all 3 ES cell lines tested (Fig. 1A) within 24h of treatment. Conversely, diminished Vimentin expression was noted by Western blotting in TC71 and ES-5838 ES cells 48h after ML327 treatment (Fig. 1A). Taken together, these results suggest that ML327 treatment induces protein expression changes consistent with partial MET features in ES cells.

We also performed light microscopy daily to observe whether morphologic changes elicited by ML327 treatment were consistent with an epithelialized phenotype. Surprisingly, we observed an increase in the proportion of rounded cells with cytoplasmic shrinking and non-adherent vesicles thought to represent apoptotic bodies (Fig. 1B) in all 3 experimental ES cells starting 48–72h following treatment with ML327 (10 μ M). The remaining cell population lacking features of apoptosis induction did not exhibit morphologic changes consistent with epithelialization. Taken together, these findings demonstrate that ML327 is capable of inducing protein expression changes consistent with MET induction, but the predominant morphologic features elicited are most suggestive of apoptosis induction.

3.2. ML327-induced MET is Associated with Apoptosis Induction

Our light microscopy observations led us to hypothesize that ML327 induces apoptosis in ES cells. We treated our 3 experimental ES cell lines with ML327 (10 μ M) for up to 4 days and found increased Caspase 3 cleavage and PARP cleavage in all 3 cell lines. ES-5838 and SK-N-MC cells demonstrated increased Caspase 3 and PARP cleavage by 2 days of treatment that persisted out to 4 days (Fig. 2A and 2C). TC71 cells demonstrated increased Caspase 3 and PARP cleavage by 1 day of treatment that persisted out to 3 days (Fig. 2B). These findings demonstrate that ML327 consistently induces the proteolytic activation of two critical apoptosis mediators in ES cells.

To validate our findings, we treated ES-5838, TC71, and SK-N-MC cells with ML327 (10 μ M) for 72 h and performed cell cycle analysis using flow cytometry with propidium iodide staining. In all 3 cell lines, ML327 treatment resulted in an increased percentage of cells in the Sub-G₀ cell cycle phase (Fig. 3). Specifically, ML327 induced a 26-fold and 12-fold increase in Sub-G₀ population in SK-N-MC and TC71 cells, respectively (Fig. 3). A more modest 4-fold induction of sub-G₀ was observed in ES-5838 cells treated with ML327 (Fig. 3C). Overall, these findings demonstrate that ML327 successfully induces apoptosis in ES cells.

P53 is a critical tumor suppressor capable of activating apoptosis in the presence of cellular stressors, including but not limited to DNA damage and oxidative stress. Activation of P53 can lead to rapid protein stabilization. We performed a time course experiment in SK-N-MC cells to evaluate whether p53 stabilization was associated with the induction of a ML327-mediated apoptosis. Similar to our prior findings in epithelial and neural-crest derived cells,

protein expression of p53 diminished during ML327-induced apoptosis (Suppl Fig. 1) [11]. These findings, together with our prior observations, suggest that ML327 likely induces apoptosis in a p53-independent manner.

3.3. ML327 Pre-Treatment Sensitizes ES Cell Lines to TRAIL-Induced Apoptosis

EMT in epithelial cancers has been shown to be associated with resistance to apoptosis inducing ligands [14,17]. E-cadherin expression has been shown to be necessary for apoptosis induction by TRAIL [17], a death-receptor ligand that initiates apoptosis via Caspase 8 cleavage (Fig. 4A). We therefore examined whether ML327 would sensitize ES cell lines to TRAIL-induced apoptosis. Three ES cell lines were pre-treated with ML327 (10 μ M) for 24 h followed by TRAIL (50ng/mL) for 6 h. ML327 pre-treated cells demonstrated increased Caspase 3 and PARP cleavage after TRAIL treatment compared to vehicle pre-treatment as well as ML327 alone (Figs. 4B–D).

ML327 has previously been shown to reduce cFLIPs expression in colon cancer cell lines, thereby sensitizing them to TRAIL-induced apoptosis [18]. We therefore analyzed the response of cFLIPs to ML327 treatment in 3 ES cell lines and found that cFLIPs protein was reduced (Figs. 4B–D). Taken together, these results suggest that MET in ES cell lines sensitizes to TRAIL-induced apoptosis in association with increased E-cadherin and reduced cFLIPs expression levels.

4. Discussion

Therapeutic resistance is a consistent feature of tumors arising from cells of mesenchymal origin and carcinomas that have undergone EMT in their disease progression [14]. We originally identified ML327 as a small molecule that is capable of inducing the expression of E-cadherin, a hallmark of epithelial cell fate, in advanced carcinoma cells of the lung and colon [10]. We have systemically expanded our observations to encompass tumors of neural crest origin, neuroblastoma, and now cells of mesenchymal origin (ES) [11]. E-cadherin is consistently upregulated by ML327 in all tested cell lines (colon, lung, breast, neuroblastoma, and ES cells) with the exception of RKO (colon) and MDA-MB-231 (breast) cancer cells, whose E-cadherin promoters are silenced by DNA hypermethylation [10,18]. Intriguingly, the predominant cellular response observed within colon and lung carcinomas is blockade of cellular migration with no observed changes in cellular viability appreciated *in vitro* and *in vivo* [10]. The predominant feature of our trials in neuroblastoma is growth arrest and necrosis with minimal induction of apoptotic markers [11]. Herein, we report a marked induction of apoptotic markers, Caspase 3 and PARP cleavage, in association with MET induction in ES cells. Overall, these findings suggest an enhanced sensitivity of non-epithelial derived tumors, such as ES cells, to ML327.

Incremental progress has been made in improving the outcomes of children afflicted with ES, prompting considerable investigation into the potential use of death-inducing ligands, such as TRAIL [4,12,19]. Of these agents, TRAIL has received much attention given its lack of toxicity to non-transformed cells, highlighting a potential therapeutic window for exploitation [14]. Rapid clearance and resistance have plagued early clinical trials using TRAIL, highlighting the need to identify novel small molecules that sensitize to TRAIL-

based therapeutics [14,20]. Reversal of mesenchymal or migratory features has been identified as one potential strategy for TRAIL sensitization [14]. Specifically, the HDAC inhibitor, MS-275, has been shown to both reverse EMT, attenuate metastasis, and sensitize breast cancer cells to TRAIL [21]. Our findings support these observations, as ML327 induces epithelial-like features and sensitizes ES cells to TRAIL-mediated apoptosis. We have previously reported the capacity of ML327 to mediate TRAIL sensitization in colon cancer cells, demonstrating this process to be in part mediated by down regulation of cFLIPs [18]. Our results are in support of our previous investigation, as we observe consistent downregulation of cFLIPs in association with ML327-mediated TRAIL sensitization. Further investigation into the therapeutic potential of the EMT reversal/MET induction agents, such as ML327, in combination with TRAIL-based strategies merits further investigation featuring studies to determine *in vivo* efficacy.

A persistent and critical deficiency of our current and previous characterizations of ML327 remains our inability to identify a direct intracellular effector of this small molecule. Modification of the structure of ML327 in ways that would facilitate affinity purification of bound proteins has resulted in loss of biological activity. We are currently undertaking alternative approaches to identify the mechanism. Identification of the direct intracellular target of ML327 offers both therapeutic promise and may provide mechanistic insights into the regulation of both EMT and therapeutic resistance to TRAIL.

In conclusion, we have validated the capacity of ML327 to elicit features of MET in ES cells. In contrast to prior characterizations in carcinoma and neural crest-derived tumors, ML327 elicits striking induction of apoptosis in all tested ES cell lines. Furthermore, partial MET induction in ES cells using ML327 sensitized ES cells to TRAIL-mediated apoptosis. Together, these findings support further *in vivo* characterization of ML327 in mesenchymal cancers, such as ES, both alone and in combination with TRAIL-based therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by grants from the National Institutes of Health (R01 DK61470), American College of Surgeons Resident Research Scholarship, and Rally Foundation for Cancer Research.

We thank Karen Martin for her aid in manuscript preparation. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource. The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404).

Abbreviations

ES	Ewing Sarcoma
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand

EMT	epithelial-to-mesenchymal transition
MET	mesenchymal-to-epithelial transition

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Highlights

- ML327 induces E-cadherin expression, an epithelial hallmark, in Ewing Sarcoma cells
- Induction of E-cadherin expression using ML327 is accompanied by apoptosis
- ML327 sensitizes Ewing Sarcoma cells to TRAIL-mediated apoptosis *in vitro*

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Figure 1. ML327 Induces MET Features in ES Cells
A. Immunoblotting demonstrates that ML327 enhanced E-cadherin expression in all tested ES cells and blocked vimentin in ES-5838 and TC71 cells. β -actin served as our protein loading control. **B.** Light microscopy (10x) demonstrates that ML327 (10 μ M) treatment increases the proportion of rounded cells with cytoplasmic shrinking and non-adherent vesicles thought to likely represent apoptotic bodies.

Figure 2. PARP and Caspase 3 Cleavage are Induced in ES Cells by ML327

A–C. Immunoblotting demonstrates the onset of Caspase 3 and PARP cleavage over the course of 4 days with or without ML327 (10 μ M). β -actin was our protein loading control.

Figure 3. ML327 Treatment Enhances DNA Fragmentation in ES Cells

Cell cycle analysis with propidium iodide was performed in ES cells after 72h of treatment with ML327 (10 μ M). **A**. Representative cell cycle analysis for TC71 ES cells. **B–D**. Stacked histograms demonstrate marked enrichment of the sub G₀ phase was observed by flow cytometry with propidium iodide staining in all three ES cell lines following 72h of ML327 treatment (10 μ M). All cell lines were completed in triplicate with representative replicates displayed.

Figure 4. ML327 Sensitizes ES Cells to TRAIL-mediated Apoptosis

A. Schematic demonstrating the role of cFLIP in antagonizing Trail-mediated apoptosis. **B–D.** Western blotting demonstrates increased TRAIL-mediated PARP and Caspase 3 cleavage in ES cells pretreated with ML327 (10 μ M). Decreased cFLIPs was observed in all tested ES cell lines. β -actin was our protein loading control.