Monensin, a polyether ionophore antibiotic, overcomes TRAIL resistance in glioma cells via endoplasmic reticulum stress, DR5 upregulation and c-FLIP downregulation

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Tumor necrosis factor-related apoptosis-induced ligand (TRAIL) is preferentially cytotoxic to cancer cells over normal cells. However, many cancer cells, including malignant glioma cells, tend to be resistant to TRAIL. Monensin (a polyether ionophore antibiotic that is widely used in veterinary medicine) and salinomycin (a compound that is structurally related to monensin and is preferentially cytotoxic to cancer cells over normal cells) are currently recognized as anticancer drug candidates. In this study, we showed that monensin effectively sensitizes various glioma cells, but not normal astrocytes, to TRAIL-mediated apoptosis; this occurs at least partly via monensin-induced endoplasmic reticulum (ER) stress, CHOP-mediated DR5 upregulation and proteasome-mediated downregulation of c-FLIP. Interestingly, other polyether antibiotics, such as salinomycin, nigericin, narasin and lasalocid A, also stimulated TRAIL-mediated apoptosis in glioma cells via ER stress, CHOP-mediated DR5 upregulation and c-FLIP downregulation. Taken together, these results suggest that combined treatment of glioma cells with TRAIL and polyether ionophore antibiotics may offer an effective therapeutic strategy.

Introduction

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor family, is an attractive anticancer agent due to its ability to induce apoptosis in a variety of tumor cell types, without affecting normal cells (1). The binding of TRAIL to cell surface death receptors can kill some cancer cells that show resistance to conventional anticancer treatments (2). However, a significant portion of human cancer cell lines and primary tumor cells can resist TRAIL-induced apoptosis through intrinsic or acquired resistance mechanisms (3).

Gliomas are among the most aggressive human cancers. Despite recent advances in multimodal therapies combining surgery, radiotherapy and chemotherapy, high-grade glioma remains fatal (4). Thus, researchers are currently attempting to develop novel therapeutic strategies against malignant gliomas. Previous studies have shown that many malignant glioma cells are resistant to TRAIL-induced apoptosis, even though they express TRAIL receptors (5). Therefore, it is hoped that the identification of safe and effective agents capable of recovering TRAIL sensitivity in glioma cells may provide a means for improving the efficacy of TRAIL-based cancer therapeutics.

Monensin, a polyether ionophore antibiotic isolated from Streptomyces cinnamoneus, is widely used in ruminant animal feeds (6). The polyether ionophore antibiotics, which include monensin (7), salinomycin (8), narasin (9) and lasalocid A (10), are believed to affect their target cells by modifying the permeability of cellular membranes to cationic metal species. Previously, monensin and salinomycin were reported to be able to overcome multidrug resistance in cancer cells (11–13) and specifically kill human cancer stem cells (13,14). Therefore, polyether ionophores are currently recognized as strong candidates for cancer chemoprevention and cancer therapy (15). However, the mechanisms underlying their anticancer activities are not yet fully understood.

In this study, we showed for the first time that combined treatment with TRAIL and subtoxic doses of various polyether ionophore antibiotics, including monensin, salinomycin, narasin and lasalocid A, synergistically induces apoptosis in various human glioma cells by inducing ER stress and CHOP upregulation. Furthermore, CHOP-mediated DR5 upregulation and proteasome-mediated c-FLIP downregulation play critical roles in monensin-induced stimulation of TRAIL-mediated apoptosis. Thus, polyether ionophore antibiotic/TRAIL cotreatment may offer an attractive strategy for the treatment of malignant gliomas.

Materials and methods

Chemicals and antibodies

Monensin, nigericin, salinomycin, bafilomycin A, chloroquine (CQ) and hydroxychloroquine (HCQ) were purchased from Sigma Chemical Corporation (St Louis, MO). Narasin and lasalocid A were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human TRAIL/Apo-2 ligand (the non-tagged 19KDa protein, amino acid 114–281) was from KOMA Biotech (Seoul, Korea). Calcein acetoxymethyl ester (calcein-AM) and ethidium homodimer (EthD-1) were from Invitrogen (Carlsbad, CA). Caspase inhibitors benzylxoxy-carbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone (z-DEVD-fmk), benzyloxy-carbonyl-Iso-Glu-(OMe)-Tyr-Asp-(OMe)-fluoromethyl ketone (z-IETD-fmk) and benzylxoxy-carbonyl-Asp-(OMe)-Glu-(OMe)-Val-Asp-(OMe)-fluoromethyl ketone (z-DEVD-fmk) were from R&D systems (Minneapolis, MN). The following antibodies were used: anti-caspase-8, caspase-3, caspase-4, protein disulfide isomerase (PDI), XIAP and KDEL (Stresgen, British Columbia, Canada); anti-Bid, anti-phospho-p65, anti-p65, anti-phospho-Elf2α, anti-Elf2α and anti-CHOP (Cell Signaling Technology, Beverly, MA); anti-poly (ADP ribose) polymerase (PARP) (Upstate Biotechnology, Lake Placid, NY); anti-cleaved PARP (Epitomics, Burlingame, CA), anti-DR5 (KOMA Biotech); anti-ATF4, anti-Bcl-2, anti-Bcl-XL and anti-DR4 (Santa Cruz Biotechnology); anti-c-FLIP (Alexis, San Diego, CA); and anti-FLAG (sigma); anti-α-tubulin (Calbiochem, San Diego, CA); and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG (HRP) (Invitrogen).

Culture of glioma cell lines and normal human astrocytes

The human malignant glioma cell lines U251MG, U87MG, A172, T98G and U251N cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (Gibco BRL, Life Technologies). The cells were incubated in 5% CO₂ at 37°C. The primary cultures of normal human astrocytes were prepared from 14 week gestation of fetal cerebrum tissues as described previously (16). Human astrocyte cultures were grown in Dulbecco’s modified Eagle’s medium with high glucose supplemented with 10% fetal bovine serum and 20 mg/ml gentamicin, subcultured every 2 weeks, and cell culture passage number of <5 were used in this study.

Measurement of cell viability

Cell viability was assessed by double labeling of cells with 2 μM calcein-AM and 4 μM EthD-1. The calcein-positive live cells and EthD-1-positive dead cells were visualized using a fluorescence microscope (Axiovert 200M, Carl Zeiss, Jena, Germany).

Abbreviations:

- Calcein-AM, calcein acetoxymethyl ester
- CI, combination index
- COX IV, cytochrome c oxidase subunit IV
- CQ, chloroquine
- ER, endoplasmic reticulum
- EthD-1, ethidium homodimer
- GAPDH, glyceraldehyde-3-phosphate dehydrogenase
- GOLPH2, Golgi phosphoprotein 2
- Hormone 3-phosphate dehydrogenase
- HRP, horseradish peroxidase
- IAP, inhibitor of apoptosis protein
- MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
- NFκB, Nuclear factor kappa B
- PCR, polymerase chain reaction
- PDI, protein disulfide isomerase
- PPI, protein phosphatase inhibitor
- RT–PCR, reverse transcription–PCR
- TRAIL, tumor necrosis factor-related apoptosis-induced ligand

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Cells were washed in phosphate-buffered saline (PBS) and lysed in boiling sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer [62.5 mM Tris (pH 6.8), 1% sodium dodecyl sulfate, 10% glycerol and 5% β-mercaptoethanol]. The lysates were boiled for 5 min, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to an Immobilon membrane (Millipore, Bedford, MA). After blocking non-specific binding sites for 1 h by 5% skim milk, membranes were incubated for 2 h with specific antibodies. Membranes were then washed three times with TNET buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.05% Tween20] and incubated further for 1 h with HRP-conjugated anti-rabbit or anti-mouse. Visualization of protein bands was accomplished using enhanced chemiluminescence (Amersham Life Science, Buckinghamshire, UK).

Immunoblotting

Flow cytometry of DR5

Cells were analyzed for the surface expression of DR5 by indirect staining with primary goat anti-human DR5 (R&D Systems), followed by fluorescent isothiocyanate-conjugated rabbit anti-goat IgG (Sigma). Briefly, U251MG cells (1 × 10⁶) were stained with 200 μl PBS containing saturating amounts of anti-DK5 antibody on ice for 30 min. After incubation, cells were washed twice with PBS and reacted with fluorescent isothiocyanate-conjugated rabbit anti-goat IgG on ice for 30 min. After washing with PBS, the expressions of these death receptors were analyzed by a fluorescence-activated cell sorter (fluorescence-activated cell sorting; Becton Dickinson and Co.).

Reverse transcription–PCR analysis

Total RNA was extracted from U251MG cells using the TRIzol reagent (Invitrogen). Reverse transcription–PCR (RT–PCR) was done, following the manufacturer’s protocol (Takara Shuzo Co., Otsu, Shiga, Japan). The cDNAs were amplified by PCR (94°C for 30 s, 60°C for 30 s and 72°C for 1 min) with Taq DNA polymerase (Takara Shuzo Co.). Conditions for final analysis were chosen when amplification of messenger RNA (mRNA) was in the middle of the exponential amplification phase for 0.25 μM monensin. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the sense primer 5′-CGGCAATCAGCCCGACAGTTT-3′ and the antisense primer 5′-CGGCGATACGCCACAGTTT-3′ (corresponding to a 310 bp region of GAPDH) were used; for CHOP, the sense primer 5′-CAACCTCAGAATGACGAGTA-3′ and the antisense primer 5′-CTGATGCTCCAATGTTGTCAT-3′ (corresponding to a 536 bp region of CHOP) for human DR5, the sense primer 5′-GTGCTCCTGATCACCAAC-3′ and the antisense primer 5′-CTGGAACTGTGACCTCCTGAT-3′ (corresponding to a 424 bp region of DR5) for c-FLIP, the sense primer 5′-CGGCTGATTAGAGCTTGG-3′ and the antisense primer 5′-GTATTAGCAGATGATGG-3′ for c-FLIP, and the sense primer 5′-CGGACTATAGAGTGCTGATGG-3′ and the antisense primer 5′-TGGCAGCATGATTGACCTG-3′ for c-FLIP. The PCR cycling conditions (30 cycles) chosen were as follows: (i) 30 s at 94°C; (ii) 45 s at 52°C for CHO, 30 s at 68°C for DR5, 30 s at 55°C for c-FLIP, 30 s at 56°C for c-FLIP, and 30 s at 60°C for GAPDH and (iii) 1 min 30 s at 72°C, with a subsequent 10 min extension at 72°C. Reaction products were analyzed on 2% agarose gels. The bands were visualized by ethidium bromide.

Small interfering RNAs

The 25 nucleotide siRNA duplexes used in this study were purchased from Invitrogen and their sequences are as follows: DR5, UACGCAUCUGUCACACGUGGC; U18, UGCUCAUAGGUGCUCGAC; BLOCK-IT Fluorescent Oligo (Invitrogen) was used as the control. Cells were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations.

Results

Monensin effectively sensitizes various glioma cells, but not normal astrocytes, to TRAIL-mediated apoptosis

Although TRAIL is considered a promising anticancer agent that does not show toxicity to normal cells (1), recent studies have revealed that numerous cancer cells, including glioma cells, are resistant to the apoptosis-inducing effects of TRAIL (5). Various ionophore antibiotics, including monensin, salinomycin and nigericin, have been reported to overcome multidrug resistance in different types of cancer cells (11–13,18), and salinomycin was recently identified as a putative cancer stem cell inhibitor (13,14). Thus, we first tested whether monensin could sensitize various glioma cell lines to TRAIL-mediated apoptosis. U251MG and U87MG cells were very resistant to monensin alone up to 100 μg/ml, A172 and T98G cells were moderately sensitive to this treatment and U251N cells were fairly sensitive (Figure 1A). Although all of these glioma cell lines were resistant to monensin up to 0.5 μM, cotreatment with monensin dose-dependently enhanced TRAIL-mediated cell death. Unlike these glioma cells, normal astrocytes were very resistant to TRAIL and/or monensin (Figure 1A), suggesting that combined treatment with monensin and TRAIL may safely and effectively kill malignant glioma cells. An isobologram analysis demonstrated that monensin and TRAIL synergistically induced cell death in U251MG, U87MG, A172, T98G and U251N cells, dose-dependent effects were determined for each compound and for one compound with fixed concentrations of another. The interaction of monensin and TRAIL was quantified by determining the combination index (CI), in accordance with the following classic isobologram (17). The equation for the isobologram is shown as CI = (D1)/Dx1 + (D2)/Dx2, where (Dx1) and (Dx2) indicate the individual dose of monensin and TRAIL required to produce an effect, and (D1) and (D2) are the doses of monensin and TRAIL, respectively, in combination that produce the same effect. From this analysis, the combined effects of the two drugs can be summarized as follows: CI < 1 indicates synergism, CI = 1 indicates summation (additive and zero interaction) and CI > 1 indicates antagonism.

Statistical analysis

All data were presented as mean ± standard deviation (SD) from at least three separate experiments. Student’s t-test was applied to evaluate the differences between treated and control groups with cell viability. Data from multiple groups were analyzed by one-way analysis of variance, followed by Bonferroni’s multiple comparison test. For all the tests, the level of significance was values of P < 0.05.
Next, we investigated whether monensin stimulates TRAIL-mediated cell death via caspase activation. Treatment of U251MG and U87MG cells with 0.25 μM monensin did not induce any proteolytic processing of caspase-8 or caspase-3 (Figure 1C). Treatment with 100 ng/ml TRAIL alone induced partial cleavage of procaspase-3 into its p20 intermediate, but further cleavage into the p17 active subunit was not observed. In addition, TRAIL alone did not induce processing of caspase-8, Bid (a specific substrate of caspase-8) or PARP (a classical substrate of caspase-3). However, cotreatment with monensin and TRAIL effectively induced the processing of caspase-8, PARP, Bid and procaspase-3 (all the way to the p17 active subunit). Pretreatment of U87MG and U251MG cells with z-V AD-fmk (a pan-caspase inhibitor) almost completely blocked the cell death induced by the combined treatment (Figure 1D). In addition, z-IETD-fmk (an inhibitor of caspase-8) and z-DEVD-fmk (an inhibitor of caspase-3) significantly and dose-dependently inhibited cell death in cotreated cells (Figure 1D). Taken together, these results demonstrate that monensin recovers TRAIL sensitivity in glioma cells via induction of caspase-mediated apoptosis.

Next, we investigated the mechanisms by which monensin sensitizes glioma cells to TRAIL-mediated apoptosis. As monensin is known to inhibit autophagy by blocking lysosomal protein degradation (19,20), we first tested whether the monensin-induced inhibition of autophagy contributes to its enhancement of TRAIL-mediated apoptosis. For this, we examined whether TRAIL-mediated apoptosis could be sensitized by other agents that inhibit autophagy at the lysosomal step, including bafilomycin A, which is a vacuolar ATPase inhibitor (21), CQ, which is a lysosomotropic agent (22) and HCQ, which is a derivative of chloroquine (20). Neither CQ nor HCQ did increase TRAIL-mediated cell death (Supplementary Figure 1A, available at Carcinogenesis Online). Bafilomycin A enhanced TRAIL-induced cell death, but to a much smaller degree than monensin. These findings prompted us to ask whether these differences in efficacy were associated with differences in autophagy-inhibiting activity. We assessed the effects of these autophagy inhibitors on the levels of the autophagy substrate proteins, p62 (23) and NBR1 (Neighbor of Brca1 gene) (24). Our results revealed that bafilomycin A, CQ and HCQ induced the accumulation of both p62 and NBR1, together with LC3 II form (Supplementary Figure 1B, available at Carcinogenesis Online). Furthermore, the proteolytic processing of cathepsin D, a major lysosomal protease (25), into its mature form (28 kDa) was more severely impaired by treatment with bafilomycin A, CQ or HCQ than by monensin treatment. These results suggest that the autophagy-inhibiting activities of bafilomycin A, CQ and HCQ may be more potent than that of monensin under our experimental conditions. Thus, the autophagy-inhibiting activity of monensin may not be critically involved in the stimulation of TRAIL-mediated apoptosis.
Monensin induces ER stress leading to CHOP-mediated DR5 upregulation

Monensin is a carboxylic ionophore that binds Na+/H+ exchanger; it can be incorporated into cholesterol-rich membranes and causes leakage of H+ from acidic organelles, including lysosomes and the Golgi apparatus (26,27). Therefore, we investigated whether the stimulating effect of monensin on TRAIL-mediated apoptosis was correlated with the physical and/or functional modulation of these organelles. To test this possibility, we treated U251MG cells with monensin and performed immunocytochemistry using antibodies against respective organelle marker proteins, including COX IV (mitochondria), GOLPH2 (Golgi apparatus), PDI (ER) and LAMP2 (lysosomes). The filamentous morphologies of the mitochondria and the punctuated morphologies of lysosomes around the nuclei were not noticeably altered (Figure 2A). Although untreated U251MG cells showed a typical perinuclear network appearance, the Golgi apparatus of monensin-treated cells was clumped and diffused. Consistent with a previous report (26), these results showed that monensin induced a marked perturbation of the Golgi apparatus. Interestingly, monensin treatment markedly enhanced the expression of an ER marker protein, PDI (28). To further test whether monensin induces ER stress, we examined the expression levels of several ER stress-associated proteins. We found that treatment of U251MG and U87MG cells with 0.25 μM monensin markedly increased the expression levels of KDEL (particularly, GRP78), phosphorylated eIF2α, ATF4, PDI and CHOP (Figure 2B). Furthermore, monensin induced the cleavage of procaspase-4 (Figure 2B), whereas it did not cleave procaspase-8 and procaspase-3 (Figure 1C). When we further examined the expression of these ER stress markers by immunocytochemistry, we found that the expression levels of both KDEL and phosphorylated eIF2α were remarkably enhanced in U251MG cells treated with 0.25 μM monensin (Figure 2C). Furthermore, monensin dramatically increased the nuclear expression of ATF4 and CHOP. Taken together, these results indicate that monensin induces ER stress.

TRAIL is known to trigger apoptotic signals by associating with DR5 at the cell surface (29). The representative ER stress inducers, thapsigargin (30) and tunicamycin (31), were previously reported to enhance TRAIL-mediated apoptosis via CHOP-induced DR5 upregulation. Therefore, we first tested whether DR5 expression at the cell surface is affected by monensin treatment. Flow cytometry showed...
that treatment of U251MG cells with 0.25 μM monensin increased the expression of DR5 at the cell surface (Figure 3A). Next, we examined whether monensin-mediated ER stress could upregulate CHOP, thereby triggering DR5 upregulation and sensitizing cells to TRAIL-mediated apoptosis. We found that an increase in CHOP protein levels preceded the monensin-induced upregulation of DR5 in U251MG and U87MG cells (Figure 3B). In addition, RT–PCR analysis showed that treatment of U251MG cells with 0.25 μM monensin increased the mRNA levels of both CHOP and DR5 (Figure 3C). To confirm the direct involvement of CHOP in this monensin-induced transcriptional activation of the DR5 promoter, we performed a luciferase assay with a reporter construct containing a DR5 promoter sequence that included the CHOP-binding site (pDR5-605), and one in which the CHOP-binding site had been mutated (pDR5-605-mCHOP). We found that monensin treatment increased the transcriptional activity of the DR5 promoter, but that this effect was blocked by mutation of the CHOP-binding site (Figure 3D).

**CHOP-mediated DR5 upregulation is critical for monensin-stimulated TRAIL-mediated apoptosis**

Next, we used a DR5/Fc chimeric protein and DR5 siRNA to test the functional significance of monensin-induced DR5 upregulation in the sensitization of cells to TRAIL-mediated apoptosis. We found that pretreatment of U251MG cells with DR5/Fc protein dose-dependently inhibited the cell death induced by monensin plus TRAIL (Figure 3E), and siRNA-mediated knockdown of DR5 expression significantly blocked this cell death (Figure 3F), suggesting that monensin-induced DR5 upregulation critically contributes to its sensitizing effect on TRAIL-mediated apoptosis. Furthermore, siRNA-mediated suppression of CHOP expression inhibited the monensin-induced increase in DR5 protein levels and attenuated the cell death induced by monensin plus TRAIL (Figure 3G). Taken together, these results suggest that CHOP is important for the enhancement of TRAIL-mediated apoptosis via induction of DR5.

**Proteasome-mediated c-FLIP downregulation is also critical for the monensin-induced sensitization of TRAIL-mediated apoptosis**

We further tested whether monensin stimulates TRAIL-mediated apoptosis by modulating other apoptotic regulators. We observed marked downregulation of c-FLIP (including c-FLIP L and c-FLIP S) in U251MG and U87MG cells treated with 0.25 μM monensin (Figure 4A). In contrast, the protein levels of DR4, XIAP, Bcl-2, Bcl-xl and phosphorylated p65 were not noticeably affected by monensin treatment (Figure 4A). Because the protein levels of both c-FLIP L and c-FLIP S were reduced by monensin, we tested whether monensin downregulates c-FLIP at the transcriptional level. However, RT–PCR analysis showed that the mRNA levels of c-FLIP L and c-FLIP S were not noticeably reduced by monensin (Figure 4B). As the expression of c-FLIP was reported to be controlled by the proteasome (32), we examined the effect of a proteasomal inhibitor on the protein levels of c-FLIP in the absence or presence of monensin. Pretreatment of U251MG cells with MG132 dose-dependently increased the protein levels of c-FLIP in both the absence or presence of monensin (Figure 4C). We found that overexpression of c-FLIP L, but not c-FLIP S, effectively attenuated the cell death induced by monensin plus TRAIL (Figure 4D). Taken together, these results suggest that proteasome-mediated downregulation of c-FLIP, in particular c-FLIP L, contributes to the sensitizing effect of monensin on TRAIL-mediated apoptosis.

**Other polyether carboxylic ionophore antibiotics also sensitize glioma cells to TRAIL-mediated apoptosis via DR5 upregulation and c-FLIP downregulation**

Finally, we examined whether other polyether ionophores with chemical structures similar to those of monensin could also sensitize glioma cells to TRAIL-mediated apoptosis (Figure 5A). Interestingly, treatment of U251MG cells with subtoxic doses of various ionophore antibiotics (e.g. nigericin, salinomycin, narasin and lasalocid A) dose...
Fig. 3. CHOP-mediated DR5 upregulation is critical for the sensitization of TRAIL-mediated apoptosis. (A) U251MG cells were untreated or treated with 0.25 μM monensin for 20h. Cells were subjected for the flow cytometry to measure the cell surface expression of DR5. (B) Cells were treated with 0.25 μM 1923.
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M.J. Yoon et al. (Figure 5B). In addition, cotreatment with these ionophore antibiotics completed the TRAIL-mediated partial proteolytic processing of procaspase-3, leading to generation of the active p17 subunit and PARP cleavage (Figure 5C). Nigericin, salinomycin, narasin and lasalocid A also commonly increased the expression levels of proteins associated with monensin for indicated time points, and cell extracts were prepared for western blotting of CHOP and DR5. Western blot of α-tubulin served as a loading control. (C) Total RNAs were isolated from U251MG cells treated with 0.25 μM monensin for the indicated time points. RT–PCR analysis of CHOP, DR5 and GAPDH was performed. (D) U251MG cells were transfected with pDR5-WT (pDR5-605) or CHOP-mutated pDR5-mCHOP (pDR5-605-mCHOP) and then treated with 0.25 μM monensin and 100 ng/ml TRAIL for 24h. Cellular viability was assessed using calcein-AM and EthD-1. Columns, average of three independent experiments; bars, SD; *P < 0.005 versus control; #P < 0.005 versus monensin plus TRAIL.

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Enhancement of TRAIL-mediated apoptosis by monensin

ER stress, including KDEL, phosphorylated eIF2α, ATF4, CHOP and processed caspase-4 (Figure 6A), indicating that polyether ionophore antibiotics commonly induce ER stress. Based on this, we investigated whether they could stimulate TRAIL-mediated apoptosis via the same mechanism as monensin. We found that these polyether ionophores time- and dose-dependently induced DR5 upregulation and c-FLIP downregulation (Figure 6B and C), suggesting that these polyether ionophores share a common mechanism with monensin to enhance TRAIL-mediated apoptosis in glioma cells (Figure 6D).

![Fig. 5.](https://academic.oup.com/carcin/article-abstract/34/8/1918/2463350)

Taken together, our results indicate that treatments combining these polyether ionophore antibiotics plus TRAIL may provide an effective therapeutic strategy for killing malignant glioma cells.

**Discussion**

TRAIL is an attractive candidate for cancer treatment due to its selective apoptosis-inducing activity in various cancer cells (1). However, numerous cancer cells, including malignant glioma cells, have
been reported to show resistance to the cytotoxic effects of TRAIL, even though they express the TRAIL receptor, DR5 (5). Therefore, researchers are currently seeking to identify agents that may effectively increase the sensitivity of cancer cells to TRAIL-induced apoptosis. Monensin, which is synthesized in *S. cinnamonensis*, is a polyether antibiotic that acts as a carboxylic ionophore to bind Na⁺/H⁺ exchanger (6,33). The ionophore antibiotics, including monensin, salinomycin, and nigericin, are potent TRAIL sensitizers in glioma cells. Recently, monensin, salinomycin and nigericin were shown to induce growth arrest and/or apoptosis in human cancer cells of different origins (35–37) and to reverse the multidrug resistance phenotype of cancer cells (11–13,38). Furthermore, salinomycin and nigericin have been shown to selectively kill cancer stem cells (13,14), which can display numerous mechanisms of resistance to chemotherapeutic drugs and irradiation therapy, allowing them to survive current therapies and to initiate long-term tumor recurrence and metastasis (39). Thus, these polyether ionophore antibiotics may be considered as novel anticancer agents that not only deplete stem cells but also induce apoptosis or overcome multiple mechanisms of resistance to apoptosis in human cancer cells (15). Continued investigation of the action mechanisms of polyether antibiotics is warranted, in the hopes of identifying more effective therapeutic strategies for treating various cancers resistant to the existing therapies.

In this study, we found that subtoxic doses of monensin and TRAIL synergistically induced apoptosis in malignant glioma cells and explored the mechanisms underlying monensin-enhanced TRAIL-mediated apoptosis. Because monensin is known to disrupt the function of acidic organelles, including lysosomes and the Golgi apparatus (27,28), we first tested the possibility that the autophagy-inhibiting activity of monensin may be involved in sensitizing cells to TRAIL-mediated apoptosis. However, we found that this sensitization was not mimicked by other agents that inhibit autophagy at the lysosomal degradation step, including bafilomycin A, CQ and HCQ. The morphologies of lysosomes, which were detected by the immunocytochemical analysis of LAMP2, were not noticeably affected by monensin treatment, but the structures of the Golgi apparatus, which were detected by the immunocytochemical analysis of GOLPH2, were swollen and impaired, consistent with a previous report (26). Most strikingly, the expression of PD, an ER resident protein (40), was markedly increased by monensin. Subsequent experiments showed that the expression levels of other ER stress marker proteins, including KDEL, phosphorylated eIF2α, ATF4 and CHOP, were also remarkably increased, suggesting that monensin induces ER stress. The CHOP gene shows extremely high induction during ER stress, which is associated with DR5 upregulation and c-FLIP downregulation. (6,33). In this study, we showed that the DR5 upregulating agents, silibinin (43) and arsenic trioxide (44) effectively stimulate TRAIL-mediated apoptosis in glioma cells via a process that critically depends on CHOP (43,44). In this study, we found that monensin increased the protein level of DR5, but not DR4, in glioma cells. A DR5 blocking antibody or siRNA-mediated DR5 knockdown effectively blocked the cell death induced by monensin plus TRAIL, confirming the functional significance of DR5 upregulation in monensin-stimulated TRAIL-mediated apoptosis. In addition, the following evidence indicates...
that CHOP mediates DR5 upregulation at the transcriptional level: (i) induction of CHOP preceded the upregulation of DR5 at both the mRNA and protein levels following monensin treatment, (ii) mutation of the CHOP-binding site in the DR5 promoter abrogated monensin-induced activation of the DR5 promoter and (iii) siRNA-mediated knockdown of CHOP inhibited monensin-induced DR5 upregulation, contributing to the attenuation of monensin-stimulated TRAIL-mediated cell death.

The detailed mechanisms through which monensin induces ER stress remain to be clarified. The main action of monensin is the exchange of protons for Na⁺; the drug triggers osmotic swelling of Golgi compartments by virtue of its membrane-associated effect as a cationophore (26). In the Golgi apparatus, secretory proteins undergo various kinds of processing (e.g. modification of sugar moieties and cleavage of peptide bonds) and are sorted to their final destinations (27). Monensin treatment inhibits the post-Golgi transport of secretory proteins and lipids, and blocks modification of sugar chains in the Golgi apparatus, such as the incorporation of galactose, sialic acid and fucose (45). These pleiotropic effects are thought to ensue from slowed trafficking across the Golgi stack and inefficient enzymatic processing in swollen compartments (26). Reductions in ER-to-Golgi protein trafficking potentially contribute to ER stress. For example, brefeldin A, which is one of the most thoroughly investigated Golgi-disrupting agents (26), has been shown to cause protein accumulation in the ER by inhibiting ER–Golgi transport (46) and it was reported to activate the CHOP promoter (47). Therefore, we cannot exclude the possibility that monensin-induced failures of appropriate protein modification in the Golgi complex could result in the accumulation of unfolded proteins in the ER, triggering ER stress.

Although c-FLIP is expressed as multiple splice variants at the transcript level, two main forms are expressed at the protein level: c-FLIP short form (c-FLIPs), a 28 kDa protein that contains two death effector domains, and c-FLIP long form (c-FLIPL), a 55 kDa protein that has two death effector domains and an inactive caspase-like domain (48). c-FLIP is known to inhibit death receptor-mediated apoptosis via inhibition of caspase-8 activation, and to block the mitochondria-mediated apoptosis induced by chemotherapeutic drugs in cancer cells (48,49). In this study, we found that the protein levels of both c-FLIPS and c-FLIPL were downregulated in response to monensin treatment. Recently, c-FLIP protein levels were shown to be correlated with TRAIL resistance in some tumor types, and c-FLIP downregulation has been implicated in chemotherapysensitized TRAIL-induced apoptosis (48,49). In our study, overexpression of c-FLIPS more effectively attenuated the cell death induced by monensin plus TRAIL compared with c-FLIPL. When we examined the mechanism underlying this monensin-induced c-FLIP downregulation, we found that monensin did not affect the mRNA levels of c-FLIPS or c-FLIPL, whereas pretreatment with the proteasome inhibitor, MG132, dose-dependently increased the protein levels of c-FLIPS and recovered the monensin-induced downregulation of c-FLIP (especially c-FLIPS). Taken together, these results indicate that proteasome-mediated c-FLIP downregulation critically contributes to monensin-facilitated TRAIL-mediated apoptosis.

Notably, we found that various other polyether ionophore antibiotics, including nigericin, salinomycin, narasin and lasalocid A, could effectively sensitize glioma cells to TRAIL-mediated apoptosis in a manner similar to monensin. Induction of ER stress, DR5 upregulation and c-FLIP downregulation by these ionophore antibiotics may be commonly critical for the recovery of TRAIL sensitivity in glioma cells (Figure 6D). Therefore, our results suggest that the sensitizing effects of these polyether ionophore antibiotics on TRAIL-mediated apoptosis may be beneficial for improving the efficacy of TRAIL-based cancer therapy.

Supplementary material
Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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