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

Mi Jin Yoon, You Jung Kang, In Young Kim, Eun Hee Kim, Jeun Ah Lim, Jun Hee Lim, Taeg Kyu Kwon, and Kyeong Sook Choi*

Department of Biomedical Sciences, Institute for Medical Sciences, Ajou University School of Medicine, Suwon, Korea and Department of Immunology, Keimyung University School of Medicine, Taegu, Korea

*To whom correspondence should be addressed. Tel: +82 31 219 4552; Fax: 82 31 219 4530; Email: kochi@ajou.ac.kr

Introduction

The tumor necrosis factor-related apoptosis-inducing 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 shows cancer stem cell-inhibiting activity) are currently recognized as anticancer drug candidates. In this study, we show 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.

Materials and methods

Abbreviations: Calcine-AM, calcine acetoxymethyl ester; CI, combination index; COX IV, cytochrome c oxidase subunit IV; CQ, chloroquine; ER, endoplasmic reticulum; EthD-1, etidium homodimer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GOLPH2, Golgi phosphoprotein 2; HCO, hydroxychloroquine; HRP, horseradish peroxidase; LAMP2, lysosome-associated membrane protein-2; mRNA, messenger RNA; PARP, poly (ADP ribose) polymerase; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; RT–PCR, reverse transcription–PCR; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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/Apo2 ligand (the non-tagged 19kDa protein, amino acid 114–281) was from KOMA Biotech (Seoul, Korea). Calcine acetoxymethyl ester (calcine-AM) and ethidium homodimer (EthD-1) were from Invitrogen (Carlsbad, CA). Caspase inhibitors benzoyl-carbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone (z-VAD-fmk), benzoyl-carbonyl-Isoglu-(OMe)-Tyr-Asp-(OMe)-fluoromethyl ketone (z-IETD-fmk) and benzoyl-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), XLP and KDEL (Stressgen, 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); anti-Flag (sigma); anti-α-tubulin (Calbiochem, San Diego, CA); horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG HRP (Invitrogen).

Carcinogenesis vol.34 no.8 pp.1918–1928, 2013
doi:10.1093/carcin/bgt137
Advance Access publication June 3, 2013

© The Author 2013. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Carcinogenesis
Immunoblotting

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-P 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% Tween 20] 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).

Immunocytochemistry

After treatments, cells were fixed with acetone/methanol (1:1) for 5 min at −20°C and blocking in 5% normal horse serum in PBS for 30 min. Fixed cells were incubated overnight at 4°C with primary antibodies [anti-cytochrome c oxidase subunit IV (COX IV 1, 1:500, rabbit; GeneTex, Irvine, CA), antilysosome-associated membrane protein-2 (LAMP2, 1:200, mouse; Santa Cruz Biotechnology), anti Golgi phosphophosphoprotein 2 (GOLPH2, 1:500; rabbit; Genetex), and anti-PDI (1:500, rabbit; Stressgen), anti-KDEL (1:500, mouse; Stressgen), anti-phospho-eIF2α (1:100, rabbit; Cell Signaling), anti-ATF4 (1:100, rabbit; Santa Cruz Biotechnology) and anti-CHOP (1:500, mouse; Cell Signaling)] diluted in PBS and then washed three times in PBS and incubated for 1 h at room temperature with anti-rabbit or anti-mouse Alexa Fluor 594 (1:500; Molecular Probes). Slides were mounted with ProLong Gold antifade mounting reagent (Molecular Probes), and cell staining was visualized with a fluorescence microscope (Axiovert 200M, Carl Zeiss).

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 fluorescein 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 and reacted with fluorescein 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′-CGGGCTACTACGGCCCACTGTTT-3′ and the antisense primer 5′-CGGCCATCAGCAGCCACAGTTT-3′ (corresponding to a 310 bp region of GAPDH) for CHOP, the sense primer 5′-CAACGGCAGAATGGCAGCTA-3′ and the antisense primer 5′-CTGATGCTCCCAATTTGTCAT-3′ (corresponding to a 536 bp region of CHOP) for human DR5, the sense primer 5′-GGCTGCTCTGATCACCCAAC-3′ and the antisense primer 5′-CTGGACAACTTGGACCTCATGTC-3′ (corresponding to a 424 bp region of DR5) for c-FLIPL, the sense primer 5′-CGGGCTACTACGGCCCACTGTTT-3′ and the anti-sense primer 5′-GGATGTTCCGAGATCTCATGTT-3′ (corresponding to 655 bp region of c-FLIPL) and for c-FLIPp, the sense primer 5′-CCGACTACTAGGGCTGATGG-3′ and the antisense primer 5′-ATGGGGCACTGTCGGATCT-3′ (corresponding to 561 bp region of c-FLIPp) were used. The PCR cycling conditions (30 cycles) chosen were as follows: (i) 30 s at 94°C; (ii) 45 s at 52°C for CHOP, 30 s at 68°C for DR5, 30 s at 55°C for c-FLIPL, 30 s at 56°C for c-FLIPp 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, UACGCAUCUGGCUCUGCUCCGCU; CHOP, UCACCAUCUCGUCACAGAGCU; 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.

Plasmids, transfection and luciferase assay

The pD5R-WT [containing DR5 promoter sequence (~605/543)] was gifted from Dr T.Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). Point mutations of the CHOP-binding sites to the DR5-WT promoter were generated by a two-step PCR method using the following primers: mCHOP (5′-CTTCCGAGGAGGTAGTGACCA to 5′-CTTCCGAGGAGGTAGTGACCA), kindly provided by Dr S.I.Park, Korea Centers for Disease Control and Prevention, Seoul, Korea) to contain these sequences with the specific primers, c-FLIPp and c-FLIPL, cDNA fragments were digested with Kpn I and Xho I and subcloned into the pcDNA 3.1 (+) vector (Invitrogen), and the resulting constructs were confirmed by nucleotide sequencing. For transfection, in brief, cells were plated onto 60 mm culture dishes at a density of 3×10⁴ cells and grown overnight. Cells were transfected with 1 μg of the respective luciferase reporter construct using Lipofectamine Plus reagent (Invitrogen) following the manufacturer’s instructions. After incubation for 24 h, transfected cells were further treated with or without monensin. Luciferase activities were assayed following the manufacturer’s protocol (Promega, Madison, WI).

Isobologram analysis

To determine the effect of combination of monensin and TRAIL on 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 = (D)/[(Dx)(Dy)], where (Dx) and (Dy) indicate the individual dose of monensin and TRAIL required to produce an effect, and (Dx) and (Dy) 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 multiple comparison test. For all the tests, the level of significance was values of P < 0.05.

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 TRAIL alone up to 100 ng/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 (Figure 1B), clearly suggesting that monensin could be an effective therapeutic agent in combination with TRAIL for treating glioma, both for TRAIL-resistant glioma and TRAIL-sensitive glioma.
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-VAD-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 Braca1 gene) (24). Our results revealed that bafilomycin A, CQ and HCQ induced the accumulation of both p62 and NBR1, together with LC3 II form, more markedly than monensin (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.
Enhancement of TRAIL-mediated apoptosis by monensin

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-FLIPα and c-FLIPλ) 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 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-FLIPα in the absence or presence of monensin. In contrast, the protein levels of c-FLIPα and c-FLIPλ were not noticeably affected 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-FLIPα in the absence or presence of monensin. In contrast, the protein levels of c-FLIPα and c-FLIPλ were not noticeably affected by monensin (Figure 4B). Additionally, the inhibition of the proteasomal pathway by MG132 failed to attenuate 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-FLIPα, 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
dependently increased TRAIL-mediated cell death (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 for 12 h, lysed, and assayed for luciferase activity. Columns, average of three independent experiments; bars, SD; *P < 0.005 versus control; #P < 0.005 versus monensin. (E) U251MG cells were transfected with the control fluorescent oligonucleotide (siF-Oligo) or siRNA duplexes against CHOP, incubated for 24 h, and further treated with 0.25 μM monensin alone for 24 h. Western blotting of CHOP and DR5 was performed to confirm knockdown of CHOP by siRNA transfection (left). Equal loading of the protein samples was confirmed by western blotting of α-tubulin. To examine the effect of CHOP downregulation on monensin-sensitized TRAIL-induced apoptosis, transfected cells with the control (siF-Oligo) or CHOP siRNA were treated with 0.25 μM monensin plus 100 ng/ml TRAIL for 24 h. Cellular viability was determined using calcein-AM and EthD-1 (right). Columns, average of three independent experiments; bars, SD; *P < 0.001 versus control; #P < 0.005 versus siF-Oligo.
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).

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. cinnamomensis, is a polyether antibiotic that acts as a carboxylic ionophore to bind Na+/K+ exchanger (6,33). The ionophore antibiotics, including monensin, salinomycin, and narasin (a derivative of salinomycin isolated from S. albus) and lasalocid A (a K+/H+ ionophore produced by strains of S. lasaliensis), are widely used to promote growth in cattle and swine as prophylactic or therapeutic anticoccidial and antibacterial agents (33). Indeed, more animals have been medicated with ionophores, such as monensin, for the control of disease than any other medicinal agents in the history of veterinary medicine (34). In this study, we show that these ionophoric polyether antibiotics, including monensin, salinomycin, nigericin, narasin and lasalocid A, 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 PDI, 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 remarkably increased, suggesting that monensin induces ER stress.

Fig. 6. Various polyether ionophore antibiotics commonly induce ER stress. DR5 upregulation and c-FLIP downregulation. (A and B) U251MG cells were treated with 0.25 μM monensin, 20 μM salinomycin, 0.25 μg/ml nigericin, 4 μM narasin or 5 μg/ml lasalocid A for the indicated time points. Cell extracts were prepared for western blotting of the indicated proteins. (C) U251MG cells were treated with monensin, salinomycin, nigericin, narasin or lasalocid A at the indicated concentrations for 16 h. Cell extracts were prepared for western blotting of DR5 and c-FLIP proteins. (D) Schematic diagram of apoptotic pathway induced by the combined treatment with TRAIL and polyether ionophore antibiotics including monensin.

Given that TRAIL is known to trigger apoptosis through binding to its cell surface death receptors, DR4 and DR5 (29), the expression levels of these death receptors may be critical to determining the intensity and/or duration of TRAIL-induced apoptotic signaling. We recently 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

1926
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-FLIPLs 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-FLIP and recovered the monensin-induced downregulation of c-FLIP (especially c-FLIPL). 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/

Received January 19, 2013; revised April 12, 2013; accepted April 21, 2013.