Published in final edited form as:

J Appl Toxicol. 2018 March ; 38(3): 366–375. doi:10.1002/jat.3546.

Maduramicin induces apoptosis and necrosis, and blocks autophagic flux in myocardial H9c2 cells

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Abstract

Maduramicin, a polyether ionophore antibiotic, is widely used as an anticoccidial agent in poultry industry. It has been reported that maduramicin may cause heart and skeletal muscle cell damage, resulting in heart failure, skeletal muscle degeneration, and even death in animals and humans, if improperly used. However, the molecular mechanism behind its capability to cause death of cardiac cells is not known. Here, we show that maduramicin induced apoptosis and necrosis in rat myocardial cells (H9c2). Maduramicin did not apparently upregulate the expression of pro-apoptotic proteins (e.g. BAD, BAK, and BAX) or downregulate the expression of anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL, Mcl-1, and survivin). Interestingly, maduramicin increased the expression of DR4 and TRAIL, activating caspases 8/3 and triggering cleavage of poly ADP ribose polymerase (PARP). Also, maduramicin induced nuclear translocation of apoptosis inducing factor (AIF). Furthermore, maduramicin blocked autophagic flux, as evidenced by inducing accumulation of both LC3-II and p62/SQSTM1. Taken together, the above results suggest that maduramicin executes its toxicity in the myocardial cells at least by inducing caspase-dependent cell death through TRAIL/DR4-mediated extrinsic pathway, and caspase-independent cell death by inducing AIF nuclear translocation and blocking autophagic flux. Our findings provide a new insight into the molecular mechanism of maduramicin's toxicity in myocardial cells.

Keywords

Maduramicin; Myocardial cells; Apoptosis; DR4; AIF; Autophagy

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Conflict of interest: The authors declare no conflict of interest.

Introduction

Coccidiosis is a parasitic disease caused by *Eimeria* infection, which causes great economic loss in the poultry industry (Min, Dalloul and Lillehoj 2004, Williams 1998). The annual worldwide cost is estimated at about \$800 million (Williams 1998). Many drugs are available for the prevention and treatment of coccidiosis (Elliott, Kennedy and McCaughey 1998). However, so far, the most widely used compounds are the polyether ionophores (Elliott, Kennedy and McCaughey 1998). Maduramicin, a monovalent glycoside polyether ionophore antibiotic, is a most potent agent for prevention of coccidiosis in chickens and turkeys (target animals) (Dorne, Fernandez-Cruz, Bertelsen, Renshaw, Peltonen, Anadon, Feil, Sanders, Wester and Fink-Gremmels 2013, Liu, Hermann, Downey, Prosser, Schildknecht, Palleroni, Westley and Miller 1983). However, it has been observed that maduramicin can be toxic in both chickens and turkeys at high doses (>10 ppm) (Dorne, Fernandez-Cruz, Bertelsen, Renshaw, Peltonen, Anadon, Feil, Sanders, Wester and Fink-Gremmels 2013, Singh and Gupta 2003). Besides, clinically maduramicin-induced toxicity has been more frequently reported in cattle, sheep and pigs (non-target animals) fed with the broiler litter as a source of protein and minerals (Bastianello, Fourie, Prozesky, Nel and Kellermann 1995, Fourie, Bastianello, Prozesky, Nel and Kellerman 1991, Sanford and McNaughton 1991, Shimshoni, Britzi, Pozzi, Edery, Berkowitz, Bouznach, Cuneah, Soback, Bellaiche, Younis, Blech, Oren, Galon, Shlosberg and Perl 2014, Shlosberg, Harmelin, Perl, Pano, Davidson, Orgad, Kali, Bor, Van Ham, Hoida, Yakobson, Avidar, Israeli and Bogin 1992, Shlosberg, Perl, Harmelin, Hanji, Bellaiche, Bogin, Cohen, Markusfeld-Nir, Shpigel, Eisenberg, Furman, Brosh, Holzer and Aharoni 1997). In addition, there are also some cases of accidental poisoning with maduramicin in humans (Jayashree and Singhi 2011, Sharma, Bhalla, Varma, Jain and Singh 2005). Histopathologically, maduramicin can induce severe myocardial and skeletal muscle lesions (Bastianello, Fourie, Prozesky, Nel and Kellermann 1995, Fourie, Bastianello, Prozesky, Nel and Kellerman 1991, Sanford and McNaughton 1991, Sharma, Bhalla, Varma, Jain and Singh 2005, Shimshoni, Britzi, Pozzi, Edery, Berkowitz, Bouznach, Cuneah, Soback, Bellaiche, Younis, Blech, Oren, Galon, Shlosberg and Perl 2014, Shlosberg, Harmelin, Perl, Pano, Davidson, Orgad, Kali, Bor, Van Ham, Hoida, Yakobson, Avidar, Israeli and Bogin 1992, Shlosberg, Perl, Harmelin, Hanji, Bellaiche, Bogin, Cohen, Markusfeld-Nir, Shpigel, Eisenberg, Furman, Brosh, Holzer and Aharoni 1997). However, the molecular mechanism underlying the toxicity of maduramicin in myocardial cells, including mechanisms of cell death, remains unknown.

Three major types of cell death have been characterized, including apoptosis, necrosis and autophagy (Fulda, Gorman, Hori and Samali 2010). Necrosis is a passive and uncontrolled cell death, which is frequently caused by external factors such as toxins, trauma, and infection (Fulda, Gorman, Hori and Samali 2010). Necrosis is characterized by cell swelling and lysis with subsequent release of cellular content into the microenvironment, causing the inflammatory response (Fulda, Gorman, Hori and Samali 2010). In contrast, apoptosis is a kind of programmed cell death, which can be induced by a wide variety of stimuli such as nutrient deficiency, growth factor withdrawal, DNA damage, and heat shock (Fulda, Gorman, Hori and Samali 2010). Morphological features of apoptosis include cell shrinkage, membrane blebbing, DNA fragmentation, and chromatin condensation (Fulda, Gorman,

Hori and Samali 2010). Apoptosis can be triggered via caspase-dependent and -independent mechanisms (Fuchs and Steller 2011). Activation of caspase cascade can be initiated through the intrinsic or mitochondrial pathway and/or the extrinsic or death receptor pathway (Fulda and Debatin 2006). Of note, in response to certain insults, apoptosis inducing factor (AIF) can translocate from mitochondria to nucleus, resulting in caspase-independent apoptosis by causing DNA fragmentation and chromatin condensation (Sevrioukova 2011).

Autophagy is another kind of programmed cell death, including macroautophagy, microautophagy and chaperone-mediated autophagy (Ohsumi 2014). Macroautophagy (referred to as autophagy in this study) is a self-degradative process to recycle cellular components (Ohsumi 2014). Autophagy is a double-edged sword in a cell, which can either protect cells or contribute to cell damage (Ohsumi 2014). The process of autophagy includes the engulfment of cargo within the autophagosome, the delivery of cargo to the lysosome by fusion of autophagosomes with lysosomes, the degradation of cargo, and the release of the resulting macromolecules back into the cytosol (Ohsumi 2014). Microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3) and sequestosome 1 (p62/SQSTM1) are the most widely monitored proteins related to autophagy (Klionsky et al. 2016). The LC3 protein is involved in phagophore formation and typically characterized as an autophagosome marker (Tanida, Ueno and Kominami 2004). When autophagy is initiated, LC3-I, a nonlipidated form of LC3, will become to LC3-II, a lipidated form, and then LC3-II recruits polyubiquitinated protein aggregates to the autophagosome (Tanida, Ueno and Kominami 2004). p62/SQSTM1 binds to LC3-II and recognizes ubiquitinated cargo proteins for degradation (Moscato and Diaz-Meco 2009). If the autophagic flux (or autophagic degradation activity) is blocked, both LC3-II and p62/SQSTM1 are accumulated (Klionsky et al. 2016). Blockage of autophagic flux contributes to cell death or necrosis (Santin, Sicard, Vigneron, Guilbeau-Frugier, Dutaur, Lairez, Couderc, Manni, Korolchuk, Lezoualc'h, Parini and Mialet-Perez 2016, Sarkar, Zhao, Aungst, Sabirzhanov, Faden and Lipinski 2014).

Here, for the first time, we show that maduramicin induces caspase-dependent and -independent apoptosis as well as necrosis in rat myocardial H9c2 cells. This is related to activating TRAIL/DR4-caspases 8/3 mediated extrinsic pathway, promoting AIF nuclear translocation, and blocking autophagic flux in H9c2 cells.

Material and Methods

Materials

Maduramicin ammonium (molecular weight = 934.16, purity > 97%, by HPLC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (5 mg/ml), aliquoted and stored at -20°C. Dulbecco's modified Eagle's medium (DMEM) and 0.05% trypsin-EDTA were obtained from Mediatech (Manassas, VA, USA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA, USA). CellTiter 96[®] AQueous One Solution Cell Proliferation Assay Kit was from Promega (Madison, WI, USA). CF488A-Annexin V and Propidium Iodide (PI) Apoptosis Assay Kit was purchased from Biotium (Hayward, CA, USA). Enhanced chemiluminescence solution was from Perkin-Elmer Life Science (Boston,

MA, USA). The following antibodies were used: cleaved PARP, cleaved caspase 3, cleaved caspase 8, cleaved caspase 9, LC3A/B (Cell Signaling, Beverly, MA, USA), FasL, Fas/CD95, TNF α , TNFR1, TRAIL, DR4, DR5, FLIP S/L, FADD, TRADD, RIP, AIF, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p62/SQSTM1 (Novus Biologicals, Littleton, CO, USA), goat anti-mouse IgG-horseradish peroxidase (HRP), goat anti-rabbit IgG-HRP (Pierce, Rockford, IL, USA), and goat anti-mouse IgG1-Alexa Fluor[®] 594 (Life Technologies, Carlsbad, CA, USA).

Cell line and culture

Rat myocardial myoblasts (H9c2, #CRL-1446, American Type Culture Collection, Manassas, VA, USA) were grown in antibiotic-free high glucose (4.5 g/L) DMEM supplemented with 10% FBS and 2 mM glutamine, in a humidified incubator of 5% CO₂ at 37°C. The cells were split at 1:4 and sub-cultured every 3 days.

One solution assay

Cell viability was evaluated using CellTiter 96[®] AQueous One Solution Cell Proliferation Assay Kit (Promega), as described (Chen, Gu, Singh, Shang, Barzegar, Jiang and Huang 2014). Briefly, H9c2 cells suspended in the growth medium were seeded in a 96-well plate at a density of 5×10^3 cells/well (in triplicates) and grown overnight. The next day, maduramicin (0–1 μ g/ml) was added. After incubation for 24, 48 or 72 h, each well was added 20 μ l of one solution reagent and incubated for 1 h. Cell viability was determined by measuring the optical density (OD) at 490 nm using a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Wellesley, MA, USA). Treatment with the vehicle (DMSO) alone served as a control.

Trypan blue exclusion assay

Cell death was evaluated by the trypan blue exclusion assay as described (Chen, Gu, Singh, Shang, Barzegar, Jiang and Huang 2014). Briefly, cells were seeded in 100-mm dishes at a density of 3×10^5 cells per dish in the growth medium and grown overnight. The next day, maduramicin (0–1 μ g/ml) was added. Following treatment with maduramicin for 24, 48 or 72 h, the cells (including floating and attached cells) were harvested, pelleted, and resuspended in 1 ml of PBS. Then, 1 part of cell suspension was incubated with 1 part of 0.4% trypan blue solution (Sigma) for 3 min at room temperature. Finally, 10 μ l of the trypan blue/cell mixture was applied to a hemacytometer, and the unstained (viable) and stained (nonviable) cells were counted separately under a microscope. For each treatment, at least 300 cells (total) were counted, and the percentage of the stained cells was calculated.

Apoptosis assay

Apoptosis assay was performed, as described previously (Chen, Gu, Singh, Shang, Barzegar, Jiang and Huang 2014). Briefly, H9c2 cells were seeded in 145-mm dishes at a density of 5×10^5 cells/dish in the growth medium and grown overnight. The next day, maduramicin (0–0.5 μ g/ml) was added. After treatment for 72 h, cells were harvested and stained with CF488A-Annexin V and Propidium Iodide (PI) Apoptosis Assay Kit (Biotium), followed by

flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells treated with the vehicle (DMSO) alone were used as a control.

Immunofluorescence staining

The intracellular translocation of AIF was determined by immunofluorescence staining as described (Chen, Liu, Luo, Odaka, Awate, Zhou, Shen, Zheng, Lu and Huang 2012). Briefly, H9c2 cells were seeded in 6-well plates containing a glass coverslip per well at a density of 3×10^5 cells/well in the growth medium and grown overnight. Following treatment with maduramicin for 24 h, the cells were fixed with 4% paraformaldehyde for 2 h at 4°C. The coverslips were then heated in antigen retrieval buffer (0.01 mM sodium citrate buffer, pH 6.0) in a water bath at 95°C for 10 min. After rinsing three times in PBS, the cells were permeabilized in 0.1% Triton X-100 for 10 min at room temperature, and incubated with 10% normal goat serum (Santa Cruz Biotechnology) for 20 min to block nonspecific binding. Then, the cells were incubated with mouse monoclonal anti-AIF antibody (1:200, diluted in PBS containing 0.25% BSA and 0.1% saponin) overnight at 4°C, followed by incubation with goat anti-mouse IgG1-Alexa Fluor[®] 594 (1:200, diluted in PBS containing 0.25% BSA and 0.1% saponin) for 1 h at room temperature. The cells were further counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) for 2 min at room temperature. After a brief rinsing with PBS, slides were mounted in glycerol/PBS (1:1, v/v) containing 2.5% 1, 4-diazabicyclo-(2, 2, 2)octane. Cell images were taken with a fluorescence microscope equipped with a digital camera (Carl Zeiss, Jena, Germany). The integral optical density (IOD) was measured using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Newburyport, MA, USA).

GFP-LC3 puncta assay

GFP-LC3 puncta assay was performed as described (Zhou, Shen, Shang, Luo, Liu, Yan, Li and Huang 2014). Briefly, H9c2 cells, infected with recombinant adenovirus encoding GFP-tagged LC3, were seeded in 6-well plates containing a glass coverslip per well at a density of 3×10^5 cells/well in the growth medium and grown overnight. The next day, the cells were treated with maduramicin for 24 h, and then slides were mounted in glycerol/PBS (1:1, v/v) containing 2.5% 1, 4-diazabicyclo-(2, 2, 2)octane. Finally, cell images were taken with a fluorescence microscope equipped with a digital camera (Carl Zeiss, Jena, Germany).

Western blot analysis

Western blotting was performed, as described previously (Chen, Gu, Singh, Shang, Barzegar, Jiang and Huang 2014). Briefly, equivalent amounts of proteins (whole cell lysates) were separated on 8–15% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat dry milk (dissolved in PBS containing 0.05% Tween 20) for 1 h at room temperature, and then incubated with primary antibodies overnight at 4°C, followed by probing with appropriate secondary antibodies conjugated to HRP overnight at 4°C. Immunoreactive bands were visualized by using Renaissance chemiluminescence reagent (Perkin-Elmer Life Science). The blots for detected proteins were semi-quantified using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Results were expressed as mean values $\pm$ SE (standard error). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test for multiple comparisons where appropriate. A level of $P < 0.05$ was considered to be significant.

Results

Maduramicin reduces cell viability and induces cell death in H9c2 cells

The maximal concentration of maduramicin in serum can reach 70 $\mu\text{g/l}$ in broiler chickens immediately after fed 5 mg/kg of maduramicin for 14 days (Elliott, Kennedy and McCaughey 1998, Kennedy, Blanchflower and O'Dornan 1997). Also, non-target animals, such as cattle, sheep, and pigs, are more sensitive to maduramicin (Fourie, Bastianello, Prozesky, Nel and Kellerman 1991, Sanford and McNaughton 1991). To determine the toxicity of maduramicin in myocardial cells, H9c2 cells were treated with maduramicin for 24, 48 or 72 h at concentrations of 0–1 $\mu\text{g/ml}$. One solution assay revealed that maduramicin reduced cell viability in a concentration- and time-dependent manner (Fig. 1A). Similar results were detected by trypan blue exclusion assay (Fig. 1B). Of note, treatment with 1 $\mu\text{g/ml}$ maduramicin for 72 h was able to induce cell death by approximately 25%, compared to the control (~4.5%). The findings suggest that maduramicin exerts its toxicity at least by inducing cell death in the myocardial cells.

Maduramicin induces apoptosis and necrosis in H9c2 cells

There exist three major types of cell death: apoptosis, necrosis and autophagy (Fulda, Gorman, Hori and Samali 2010). To characterize the type of cell death induced by maduramicin (Fig. 1), first of all, we performed Annexin V-PI staining and flow cytometry, which can detect both apoptosis and necrosis (Crowley, Marfell, Scott and Waterhouse 2016, Krysko, Vanden Berghe, D'Herde and Vandenabeele 2008). As shown in Fig. 2, 95.4% (Q4) of H9c2 cells were viable when treated with vehicle (DMSO) for 72 h. However, treatment with 0.05 and 0.5 $\mu\text{g/ml}$ of maduramicin for 72 h reduced the percentages of viable cells to 89.4% and 70.9%, respectively. Correspondingly, treatment with maduramicin induced apoptosis and necrosis of H9c2 cells in a concentration-dependent manner. Maduramicin at 0.05 and 0.5 $\mu\text{g/ml}$ increased the proportions of apoptotic cells (Annexin V⁺/PI⁻, early apoptotic cells in Q3; Annexin V⁺/PI⁺, late apoptotic cells in Q2) by 2.0 fold and 3.7 fold, respectively, compared to the vehicle control. In addition, we noticed that treatment with maduramicin at 0.05 and 0.5 $\mu\text{g/ml}$ also drastically increased the percentages of necrotic cells (Annexin V⁻/PI⁺ cells in Q1) by 3.8 fold and 19.6 fold, respectively, compared to the control. The results indicate that maduramicin is able to induce both apoptosis and necrosis in the myocardial cells.

Maduramicin induces expression of DR4 and TRAIL, leading to activation of caspases 8 and 3 as well as cleavage of PARP in H9c2 cells

To determine whether maduramicin induces caspase-dependent apoptosis of H9c2 cells, Western blotting was performed to detect the cleavage of PARP, a hallmark of caspase-

dependent apoptosis (Fuchs and Steller 2011). As shown in Fig. 3A, treatment with maduramicin for 24 h induced a concentration-dependent cleavage of PARP in H9c2 cells. As the cleavage of PARP is a consequence of activation of caspases, we further investigated whether maduramicin activates caspases 3, an executioner caspase for apoptosis (Fuchs and Steller 2011). As expected, maduramicin was able to activate caspases 3, as judged by increased cleavage of caspase 3 by Western blot analysis (Fig. 3A).

There are two caspase-dependent apoptotic pathways, the intrinsic and the extrinsic pathways, in cells (Fuchs and Steller 2011). Firstly, we investigated whether maduramicin affects the levels of the proteins involved in the intrinsic pathway. Surprisingly, treatment with maduramicin for 24 h neither downregulated the levels of anti-apoptotic proteins, such as survivin, Mcl-1, Bcl-2 and Bcl-xL, nor upregulated the levels of pro-apoptotic proteins, including Bax, Bak and Bad (Fig. 3B). The finding was consistent with the result that maduramicin had no effect on the cleavage of caspase 9 (Fig. 3A), an initiator caspase in the intrinsic pathway (Fuchs and Steller 2011).

Next, we further examined whether maduramicin affects the protein levels of ligands/death receptors (FasL/Fas, TNF α /TNFR1, TRAIL/DR4 and TRAIL/DR5) as well as adaptors (FADD, TRADD, and RIP), which are the best-characterized proteins involved in the extrinsic pathway (Fuchs and Steller 2011). Interestingly, treatment with maduramicin for 24 h did not obviously alter the levels of FasL, Fas, TNF α , TNFR1, DR5, FADD, TRADD and RIP (receptor-interacting protein), but induced a robust expression of DR4 and TRAIL (Fig. 3C). This was consistent with the finding that maduramicin induced the cleavage of caspase 8 (Fig. 3A), an initiator caspase in the extrinsic pathway (Fuchs and Steller 2011). Collectively, the results suggest that maduramicin may induce caspase-dependent apoptosis in H9c2 cells through TRAIL/DR4-mediated extrinsic pathway.

Maduramicin induces cell death through caspase-dependent and -independent manner in H9c2 cells

To determine whether maduramicin induces cell death is fully through caspase-dependent mechanism, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-VAD-fmk), a pan-caspase inhibitor, was used. Pretreatment with z-VAD-fmk (20 μ M) for 1 h completely suppressed maduramicin-induced activation of caspase 3, as detected by Western blotting (Fig. 4A). In line with this, pretreatment with z-VAD-fmk also blocked maduramicin-induced cleavage of PARP, a substrate of caspases (Fig. 4A). However, pretreatment with z-VAD-fmk only partially prevented maduramicin-induced cell death, as detected by the trypan blue exclusion assay (Fig. 4B). Therefore, the results suggest that maduramicin induces cell death of H9c2 cells via caspase-dependent and -independent mechanisms.

Maduramicin increases AIF nuclear translocation in H9c2 cells

In response to certain insults, AIF can translocate from mitochondria to nucleus, resulting in caspase-independent apoptosis (Sevrioukova 2011). To address how maduramicin induces caspase-independent cell death in H9c2 cells, next, we studied whether maduramicin affects the protein level and the nuclear translocation of AIF. Treatment with maduramicin did not alter the cellular protein level of AIF obviously (Fig. 5A), but enhanced AIF nuclear

translocation dramatically (Fig. 5B). The result supports that maduramicin induces caspase-independent apoptosis in H9c2 cells at least by promoting AIF nuclear translocation.

Maduramicin blocks autophagic flux in H9c2 cells

Since autophagy is also involved in caspase-independent cell death (Ohsumi 2014), we further examined the autophagic status of H9c2 cells, in response to maduramicin treatment. For this, H9c2 cells, infected with recombinant adenovirus expressing GFP-tagged LC3, were treated with maduramicin for 24 h, followed by visualization under a fluorescence microscope. As shown in Fig. 6A, maduramicin-treated cells manifested a punctuated fluorescent pattern of GFP-LC3, whereas control cells showed a diffuse distribution of GFP-LC3, suggesting that maduramicin induced the formation of LC3 puncta in the cells. During autophagy, the cytoplasmic form LC3-I (18 kDa) is processed and recruited to phagophores, where LC3-II (16 kDa) is generated by site-specific proteolysis and lipidation at the C-terminus (Tanida, Ueno and Kominami 2004). Thus, the characteristic conversion from endogenous LC3-I to LC3-II is commonly used to monitor autophagosome formation (Klionsky et al. 2016). In this study, we observed that maduramicin treatment resulted in concentration-dependent increase of LC3-II (Fig. 6B), suggesting that maduramicin induces autophagosome formation.

Lysosomal degradation of autophagosomes leads to a decrease in p62/SQSTM1 levels during autophagy, so p62/SQSTM1 serves as a marker for the final execution of autophagy (Moscat and Diaz-Meco 2009). Blockage of autophagic flux correlates with increased levels of p62/SQSTM1, which contributes to cell death or necrosis (Moscat and Diaz-Meco 2009, Santin, Sicard, Vigneron, Guilbeau-Frugier, Dutaur, Lairez, Couderc, Manni, Korolchuk, Lezoualc'h, Parini and Mialet-Perez 2016, Sarkar, Zhao, Aungst, Sabirzhanov, Faden and Lipinski 2014). We further performed an autophagic flux assay by detecting the total cellular protein level of p62/SQSTM1. Western blot analysis showed that a remarkable increase of p62/SQSTM1 was detected following treatment with maduramicin for 24 h (Fig. 6B), suggesting that maduramicin blocks autophagic flux.

Discussion

Maduramicin, a most potent polyether ionophore used as an anticoccidial agent, is considerably more toxic than other ionophores (Dorne, Fernandez-Cruz, Bertelsen, Renshaw, Peltonen, Anadon, Feil, Sanders, Wester and Fink-Gremmels 2013). When maduramicin was improperly used, many toxic cases were reported in target animals (such as chickens and turkeys) and non-target animals (e.g. cattle, sheep, and pig) (Bastianello, Fourie, Prozesky, Nel and Kellermann 1995, Fourie, Bastianello, Prozesky, Nel and Kellerman 1991, Sanford and McNaughton 1991, Shlosberg, Perl, Harmelin, Hanji, Bellaiche, Bogin, Cohen, Markusfeld-Nir, Shpigel, Eisenberg, Furman, Brosh, Holzer and Aharoni 1997, Singh and Gupta 2003). Importantly, there was accidental intoxication by maduramicin in humans (Sharma, Bhalla, Varma, Jain and Singh 2005). Pathologically, maduramicin can induce focal degenerative cardiomyopathy leading to congestive heart failure, and severe skeletal muscle lesion associated with rhabdomyolysis (Bastianello, Fourie, Prozesky, Nel and Kellermann 1995, Fourie, Bastianello, Prozesky, Nel and

Kellerman 1991, Sanford and McNaughton 1991, Sharma, Bhalla, Varma, Jain and Singh 2005, Shimshoni, Britzi, Pozzi, Edery, Berkowitz, Bouznach, Cuneah, Soback, Bellaiche, Younis, Blech, Oren, Galon, Shlosberg and Perl 2014, Shlosberg, Harmelin, Perl, Pano, Davidson, Orgad, Kali, Bor, Van Ham, Hoida, Yakobson, Avidar, Israeli and Bogin 1992, Shlosberg, Perl, Harmelin, Hanji, Bellaiche, Bogin, Cohen, Markusfeld-Nir, Shpigel, Eisenberg, Furman, Brosh, Holzer and Aharoni 1997). However, to date, the molecular mechanism by which maduramicin induces degeneration of myocardial cells is not clear. Here, for the first time, we present evidence that maduramicin exerts its toxicity in rat myocardial cells (H9c2) by inducing apoptosis and necrosis and inhibiting autophagic flux.

In this study, we found that maduramicin reduced cell viability and induced cell death in myocardial cells (H9c2) in a concentration- and time-dependent manner (Fig. 1). Our data further suggested that maduramicin was able to induce both apoptosis and necrosis, but failed to induce autophagy in H9c2 cells. This is supported by the findings that treatment with maduramicin (0.05 and 0.5 µg/ml) for 72 h induced both apoptosis and necrosis significantly, as detected by Annexin V/PI staining and flow cytometry (Fig. 2). Treatment with maduramicin induced the formation of GLP-LC3 puncta (or autophagosome), but caused the accumulation of both LC3-II and p62/SQSTM1 (Fig. 6), suggesting that autophagy execution was, in fact, blocked by maduramicin.

In the present study, we observed that maduramicin was able to induce apoptotic cell death through the extrinsic pathway. Treatment with maduramicin for 24 h induced a robust protein expression of the death receptor DR4 and the ligand TRAIL (Fig. 3C). Activation of the extrinsic pathway resulted in activation of caspases 8 and 3, increasing the cleavage of PARP (Fig. 3A). We further found that z-VAD-fmk (a pan-caspase inhibitor) completely blocked maduramicin-induced expression of cleaved caspase 3 and cleaved PARP, but only partially prevented maduramicin induced cell death in H9c2 cells (Fig. 4). The interesting data imply that maduramicin may induce apoptosis of H9c2 cells through caspase-dependent and -independent mechanisms. Mechanistically, this might be attributed to the fact that maduramicin was able to induce AIF nuclear translocation in H9c2 cells (Fig. 5B). Apparently, this mechanism is not cell line dependent, since increased translocation of AIF from cytoplasm to nucleus was also observed in skeletal muscle cells (C2C12) exposed to maduramicin (data not shown).

Here we found that maduramicin was able to induce profound necrosis in myocardial cells (Fig. 2). Recently, programmed necrosis or necroptosis has been identified (Sun and Wang 2014). Necroptosis is activated by receptor interacting kinases 1/3 (RIP1/3), and inhibited by caspases, particularly caspase 8 that can proteolytically cleave and inactivate RIP1 and RIP3 (Oberst, Dillon, Weinlich, McCormick, Fitzgerald, Pop, Hakem, Salvesen and Green 2011, Sun and Wang 2014). Also, TNFα, TNFR, FADD, and TRADD play important role in the regulation of necroptosis (Sun and Wang 2014). In this study, we observed that maduramicin did not obviously alter the protein expression of RIP, TNFα, TNFR, FADD, and TRADD. Furthermore, maduramicin did not inhibit caspases 3/8/9. On the contrary, activation of caspases 3/8 was detected (Fig. 3). Thus, whether maduramicin induces necroptosis remains to be determined. Recently, it has been shown that overexpression of monoamine oxidase-A in cardiomyocytes induces the generation of reactive oxygen species (ROS), which blocks

In conclusion, this study has demonstrated that maduramicin induced apoptosis and necrosis in H9c2 myocardial cells. Mechanistically, maduramicin induced apoptosis through TRAIL/DR4-mediated extrinsic pathway, leading to activation of caspases 8 and 3 as well as cleavage of PARP. Maduramicin also induced caspase-independent apoptosis by increasing AIF nuclear translocation. In addition, maduramicin blocked autophagic flux in H9c2 cells, which might contribute to necrosis. Our findings provide a new insight into the molecular mechanism of the capability of maduramicin to cause death of myocardial cells.

Acknowledgments

This work was supported in part by the grants from National Natural Science Foundation of China (No. 31672612, S.J.), NIH (NCI CA115414; S.H.), American Cancer Society (RSG-08-135-01-CNE; S.H.), Nanjing Agricultural University, and the Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center.

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Fig. 1. Maduramicin reduces cell viability and induces cell death in H9c2 cells. H9c2 cells (plated in triplicates) were treated with maduramicin at indicated concentrations for 24, 48 or 72 h, followed by one solution assay (A) and trypan blue exclusion assay (B). Data represents mean $\pm$ SE ($n = 3$, corresponding to three independent experiments). ** $P < 0.01$, difference with the control group.

Fig. 2. Maduramicin induces apoptosis and necrosis in H9c2 cells. H9c2 cells were treated with maduramicin for 72 h at indicated concentrations, followed by Annexin V-PI staining and flow cytometry. (A) Histograms from a representative experiment show the apoptotic and necrotic effects of maduramicin on H9c2 cells. The percentages of necrotic, late apoptotic, early apoptotic, and viable cells are displayed in Q1, Q2, Q3 and Q4, respectively. (B) Bar graphs show that maduramicin induced apoptosis and necrosis of H9c2 cells in a concentration-dependent manner. Quantitative results for apoptosis (Q2 + Q3) (Left panel) and necrosis (Q1) (Right panel) are displayed as fold change compared with control. Data represents mean $\pm$ SE (n = 3). ** $P < 0.01$, difference with the control group.

Fig. 3. Maduramicin upregulates the expression of DR4 and TRAIL, leading to activation of caspases 8 and 3 as well as cleavage of PARP in H9c2 cells. H9c2 cells were treated with maduramicin for 24 h at indicated concentrations, followed by Western blotting with indicated antibodies. GAPDH was used for loading control. Representative blots are shown. Similar results were observed in at least 3 independent experiments.

Fig. 4.

Maduramicin induces caspase-dependent and -independent apoptosis in H9c2 cells. H9c2 cells, pretreated with or without z-VAD-fmk (20 μM) for 1 h, were treated with maduramicin for 24 h at indicated concentrations, followed by Western blotting with indicated antibodies (A), or for 48 h at indicated concentrations, followed by trypan blue exclusion assay (B). GAPDH was used for loading control. Representative blots are shown. Similar results were observed in at least 3 independent experiments. Data represents mean $\pm$ SE ($n = 3$). ** $P < 0.01$, difference with the control group. ## $P < 0.01$, difference with z-VAD-fmk group.

Fig. 5. Maduramicin induces AIF nuclear translocation in H9c2 cells. (A and B) H9c2 cells were treated with maduramicin for 24 h at indicated concentrations, followed by Western blotting with indicated antibodies (A) or AIF immunofluorescence staining and DAPI staining (B). GAPDH was used for loading control. Representative blots and images are shown. Similar results were observed in at least 3 independent experiments. Scale bar: 10 µm.

Fig. 6. Maduramicin inhibits autophagic flux in H9c2 cells. (A) H9c2 cells, infected with recombinant adenovirus expressing GFP-tagged LC3, were treated with maduramicin for 24 h at indicated concentrations, followed by taking images under a fluorescence microscope. Representative images are shown. Similar results were observed in at least 3 independent experiments. Scale bar: 10 µm. (B) H9c2 cells were treated with maduramicin for 24 h at indicated concentrations, followed by Western blotting with indicated antibodies. GAPDH was used for loading control. Representative blots are shown. Similar results were observed in at least 3 independent experiments.