Role of Prosurvival Molecules in the Action of Lidamycin toward Human Tumor Cells[1]

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Objective   Lidamycin, an enediyne antibiotic, leads to apoptosis and mitotic cell death of human tumor cells at high and low concentrations. The reason why tumor cells have distinct responses to lidamycin remains elusive. This study was to elucidate if cellular prosurvival molecules are involved in these responses.   **Methods**   Cleavage of chromatin and DNA was observed by chromatin condensation and agarose gel electrophoresis. Accumulation of rhodamine 123 in lidamycin-treated cells was assayed by flow cytometry. Cell multinucleation was detected by staining with Hoechst 33342. Western blot and senescence-associated β-galactosidase (SA-β-gal) staining were used to analyze protein expression and senescence-like phenotype, respectively.   **Results**   SIRT1 deacetylase remained unchanged in 0.5 nmol/L lidamycin whereas cleavage occurred when apoptosis was induced by lidamycin. Increased FOXO3a, SOD-1 and SOD-2 expression and transient phosphorylation of ERK were detected after exposure of human hepatoma BEL-7402 cells to 0.5 nmol/L lidamycin. High expressions of SIRT1 and Akt were found in colon carcinoma HCT116 p53 knock-out cells exposed to lidamycin. Degradation of PARP and p53 by lidamycin as a substitute for SIRT1 and Akt was confirmed with caspase inhibitor Q-VD-OPh and proteasome inhibitor MG132. Resistance to lidamycin-induced DNA cleavage was observed in breast cancer doxorubicin-resistant MCF-7 cells. This was not induced by P-glycoprotein as no accumulation of rhodamine 123 was detected in the resistant cells following exposure to lidamycin. In contrast to sensitive MCF-7 cells, a lower multinucleation rate for the resistant cells was measured following exposure to equal concentrations of lidamycin.   **Conclusions**   Cellular prosurvival molecules, such as SIRT1, Akt, SOD-1, SOD-2 and other unknown factors can influence the action of lidamycin on human tumor cells.

Key words: Lidamycin; Prosurvival molecules; SIRT1 deacetylase; Apoptosis; Mitotic cell death; Multidrug resistance

INTRODUCTION

Lidamycin, an enediyne antitumor antibiotic isolated from a fermentation broth of *streptomyces globisporus* C1027 strain, is undergoing testing in Phase II clinical trials in China[1]. As a radiomimetic drug, lidamycin damages DNA, leading to formation of single- and double-strand breaks and abasic sites due to hydrogen atom abstraction from the deoxyribonucleotide backbone[2-3]. In contrast to radiation damage, DNA strand scission induced by lidamycin (C-1027) is independent of ataxiatelangiectasia-mutated kinase (ATM) or related phosphatidylinositol 3 (PI-3)-kinases (ATR and DNA-PK) that play a very important role in damage check point[4]. Misrejoining of chromosomes and telomere repeats is greatly increased within 26 h of lidamycin incubation when compared with an equivalent growth inhibitory dose of ionizing radiation[5].

Our previous study showed that irregular apoptosis occurs in a caspase-independent way after tumor cells are exposed to lidamycin (1 μmol/L)[6], which has been confirmed by a report of a significant increase of apoptotic cells induced by this antibiotic without obvious activation of caspases as compared with other DNA-damaging antitumor agents[7]. In addition, we reported that mitotic cell death (mitotic catastrophe) occurs with senescence phenotype when hepatoma BEL-7402 and breast cancer MCF-7 cells are treated with low concentrations of lidamycin (<1 nmol/L), a result of aberrant centrosome overduplication[8-9]. Similar to other antitumor agents,

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apoptosis has been observed in HCT-116 cells treated with lidamycin through a p53 dependent pathway and partially abrogated by over-expression of anti-apoptotic protein bcl-2 partly[10]. Concentration-dependent retardation of G_1 or G_2/M phase in MCF-7 cells exposed to lidamycin is associated with the p53 status[11]. These observations suggest that cellular factors such as prosurvival molecules have to be taken into consideration in order to fully explain the unique action of lidamycin.

Cell death or survival in tumor chemotherapy is greatly influenced by the action of antitumor drugs. Induction of apoptosis and mitotic cell death with senescence-like phenotype, two main modes of cell death, may be attributed to the cytotoxic effects of antitumor drugs *in vitro* and *in vivo*[12-14]. Suppression of apoptosis mainly results from activation of the PI3K/Akt pathway in which Akt phosphorylates a number of apoptosis-related molecules, such as Bid, caspase-3, caspase-9, *etc.*[15]. Furthermore, the occurrence of apoptosis is influenced by the expression of prosurvival molecules, such as Bcl-2/Bcl-X_L and superoxide dismutases (SOD), induced by apoptotic stimuli via FOXO3a activation[16]. In addition to Akt, longevity protein SIRT1, a NAD-dependent deacetylase of the sirtuin family, plays a very important role in survival and resistance to stress stimuli in normal cells[17]. SIRT1 silencing in tumor cells initiates an apoptotic pathway, which is other than that initiated by Bcl-2 silencing[18]. Interestingly, interaction of prosurvival kinase Akt with SIRT1 via FOXO3a forms a complex regulation network of apoptosis and senescence[19].

In this study, we examined the role of prosurvival molecules in the action of lidamycin on several cell lines. Our findings clearly show that prosurvival molecules, such as SIRT1, Akt, SOD-1, and SOD-2, influence the action of lidamycin on human tumor cells by determining the modes of cell death.

MATERIALS AND METHODS

Reagents

Lidamycin was prepared as a stock solution (100 μmol/L) in saline (0.9%), stored at -20 ℃. RNase A, DMSO, Hoechst 33342, protease K, Rhodamine 123, X-gal and MG132 were purchased from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA). RPM1640 medium was obtained from Invitrogen (Carlsbad, CA, USA). General caspase inhibitor Q-VD-Oph (OPH001) was purchased from R & D Systems.

Cell Lines and Culture Condition

Human hepatoma BEL-7402 cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Human breast carcinoma sensitive and doxorubicin-resistant MCF-7 cell lines were kindly provided by Dr. Kenneth Cowan (National Cancer Institute, MD, USA). HCT116 cells were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD, USA). These cell lines were cultured in 1640 medium supplemented with 10% fetal bovine serum (Tianjin Haoyang Biotech Company, Tianjin, China), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin. The cells were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Observation of Chromatin Condensation

After treatment with lidamycin for 1 h, the cells were stained with DNA specific dye Hoechst 33342 (2 μg/mL) for 30 min at 37 ℃, and then incubated in serum-free 1640 medium. Chromatin condensation was observed under a fluorescence microscope (Nikon, Japan).

Western Blot

Cells were seeded in a 100 mm dish to approximately 60%-80% confluence prior to lidamycin treatment, and lysed in a buffer containing Tris-HCl (25 mmol/L, pH 7.5), NaCl (150 mmol/L), EDTA (2 mmol/L), glycerol (10%), glycerophosphate (10 mmol/L), sodium pyrophosphate (5 mmol/L), NaF (5 mmol/L), Na_3VO_4 (1 mmol/L), Triton X-100 (0.5%), freshly supplemented with PMSF (1 mmol/L), aprotinin (2 μg/mL) and leupeptin (2 μg/mL). Protein (20 μg) was loaded on a lane for SDS-PAGE, transferred to a PVDF membrane, and probed with corresponding antibodies. Protein signals were detected with the ECL Plus enhanced chemiluminescence reaction system according to its manufacturer's protocol (Amersham Biosciences, Indianapolis, USA).

Antibodies against poly (ADP-ribose) polymerase (PARP, 9542), Bim (4582), Akt (9272), phospho-Akt (Ser473, 9271), p38 (9212), phospho-p38 (Thr180/Tyr182, 9211), ERK (9102), phospho-ERK (Thr202/Tyr204, 9101) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-p53 (sc-126), anti-actin (sc-1616), anti-SOD-1 (sc-11407) and anti-SIRT1 (sc-15404) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against FOXO3a and phosphorylated FOXO3a (Thr32) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Mouse anti-SOD-2 (manganese superoxide dismutase) antibody was

obtained from CHEMICON.

Isolation of DNA and Agarose Gel Electrophoresis

Genomic DNA was isolated from cells treated with lidamycin (1 μmol/L) for 1 h[6]. DNA cleavage fragments were resolved by agarose gel electrophoresis (2%).

Detection of Senescence-associated β-galactosidase (SA-β-gal) Stained Cells and Multinucleated Cells

Sensitive and resistant MCF-7 cells were treated with lidamycin for 72 h and detected as described previously[8]. In brief, after fixation in formaldehyde (3%) for 5 min, the attached cells were stained overnight with X-gal at pH 6.0 for SA-β-gal activity, and then stained with Hoechst 33342 for revealing multinucleated cells (cells with more than 3 micronuclei were counted as multinucleated cells).

Accumulation of Rhodamine 123 by Flow Cytometry

Cells (2×10^5 per well) were seeded in a 6-well plate for 24 h and treated with different concentrations of lidamycin for 1 h. After incubation with fluorescence dye rhodamine 123 (5 μg/mL) at 37 ℃ for 1 h in the dark, the intracellular fluorescence intensity was detected with a BD FACScalibur flow cytometer.

RESULTS

Expression of SIRT1 and p53 in Two Modes of Cell Death Induced by Lidamycin

Our previous study revealed that mitotic cell death occurs with senescence phenotype induced by low concentration lidamycin (0.5 nmol/L) in BEL-7402 and MCF-7 cells and typical apoptosis induced by a high concentration of lidamycin (10 nmol/L)[8,10]. Apparently, tumor cells have distinct responses to lidamycin. In order to elucidate the underlying mechanisms, we chose BEL-7402 cells for further investigation. As shown in Fig. 1A, apoptosis occurred in cells exposed to lidamycin at a concentration of 2 nmol/L or higher for 24 h as judged by the cleavage of PARP to form an 89 kDa fragment, an indictor of apoptosis. However, a small amount of full length PARP (116 kDa) was also detected even when they were treated with lidamycin at a concentration as high as 100 nmol/L which is consistent with our previous observation that apoptosis could be induced in a caspase-independent way at higher concentrations of lidamycin[6]. SIRT1 cleavage occurred when the concentration of lidamycin was 10-100 nmol/L due to the activation of

caspase since SIRT1 is a substrate of activated caspase-3[20]. Because BEL-7402 cells expressed a wild type p53 protein, maximum p53 expression was accompanied with a high rate of apoptosis at 10 nmol/L lidamycin, which supports our previous findings that induction of apoptosis by lidamycin is p53 dependent to a certain extent[10].

In order to detect the alteration of SIRT1 expression, we collected the adhered cells with mitotic cell death after BEL-7402 cells were exposed to 0.5 nmol/L lidamycin for 4 , 8, 12, and 24 h, and 2, 3, and 6 d, respectively. The level of SIRT1 remained unchanged at the indicated time points (Fig. 1B). In contrast to mitotic cell death, cleavage of SIRT1 was detected in the suspended apoptotic cells, which were harvested after incubation with lidamycin for 6 d. The level of p53 expression increased slightly at 12 and 24 h and then decreased, and decreased constantly in the apoptotic cells, suggesting that SIRT1 may play a prosurvival role in the mode choice of cell death induced by lidamycin.
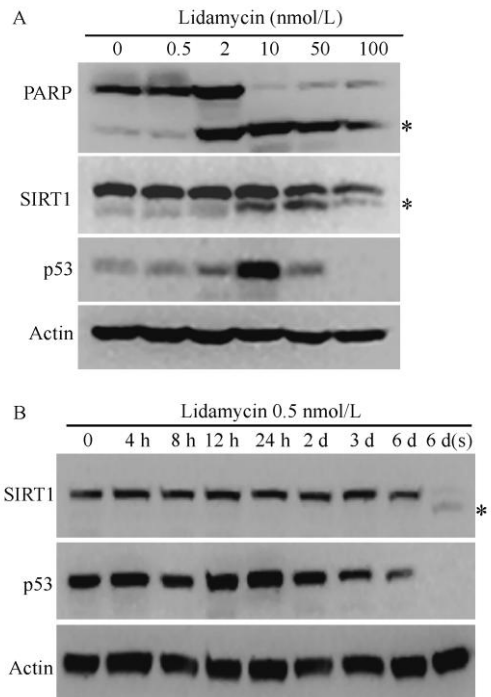

FIG. 1. Levels of SIRT1 and p53 in hepatoma BEL-7402 cells treated with various concentrations of lidamycin for 24 h (A) and after treated with 0.5 nmol/L lidamycin (B). Adherent cells were collected for Western blot analysis. *: cleaved protein fragments. A representative result from three independent experiments.

Signaling of Akt/FOXO3a and MAPK Pathways in the Cell Death Induced by Lidamycin

The observation of distinct expression profiles of SIRT1 in lidamycin-treated cells prompted us to investigate the role of other prosurvival molecules, such as Akt/FOXO3a and MAPK in the modulation of cell death. Lidamycin concentrations (0.5 nmol/L and 10 nmol/L) were selected to represent mitotic cell death and apoptosis as previously described[6,8,10]. As shown in Fig. 2, both lidamycin concentrations triggered the phosphorylation of Akt after 4 h, which then decreased at 24-72 h. The total amount of Akt remained unchanged during lidamycin treatment. The phosphorylation profile of FOXO3a, an important substrate of Akt, was similar when 0.5 nmol/L or 10 nmol/L lidamycin was used, i. e., phosphorylation of FOXO3a was significantly increased 4 h after treatment and reduced 12 h after treatment. However, the total expression level of FOXO3a in cells gradually increased upon treatment with 0.5 nmol/L lidamycin and decreased upon treatment with 10 nmol/L lidamycin.

The expression of Bim, a target protein of FOXO3a, was different. In contrast to reduction at 10 nmol/L lidamycin, its level obviously increased after exposure to 0.5 nmol/L lidamycin at the indicated time points. Elevated expression of SOD-2 (Manganese-SOD), another target of FOXO3a, appeared transiently at 4-12 h after treatment with 0.5 nmol/L lidamycin. However, the expression level of SOD-2 remained constant at 4, 8, and 12 h, and decreased at 24 h after treatment with 10 nmol/L lidamycin. The expression level of SOD-1 (cytosolic Cu/Zn-SOD) transiently increased at 4-8 h after treatment with 0.5 nmol/L and 10 nmol/L lidamycin, while the expression level of SOD-1 was higher than that of SOD-2.

Distinct activation profiles of MAPK members were observed after exposure to lidamycin (Fig. 2). Lidamycin lidamycin (0.5 nmol/L and 10 nmol/L) trigged obvious phosphorylation of ERK 8 h after treatment. The total ERK level increased at 4 h and markedly decreased at 12 h after exposure to 0.5 nmol/L lidamycin, and increased following 10 nmol/L lidamycin treatment. Although the total level of p38 expression did not change, the transient phosphorylation of p38 occurred 8 h after exposure to 0.5 nmol/L lidamycin. Phosphorylation took place 8-24 h after exposure to 10 nmol/L lidamycin, during which apoptosis occurred, suggesting that increased SOD-1, SOD-2, and FOXO3a expressions and reduction of p38 phosphorylation are also involved in the mode of cell death in response to lidamycin stimuli.
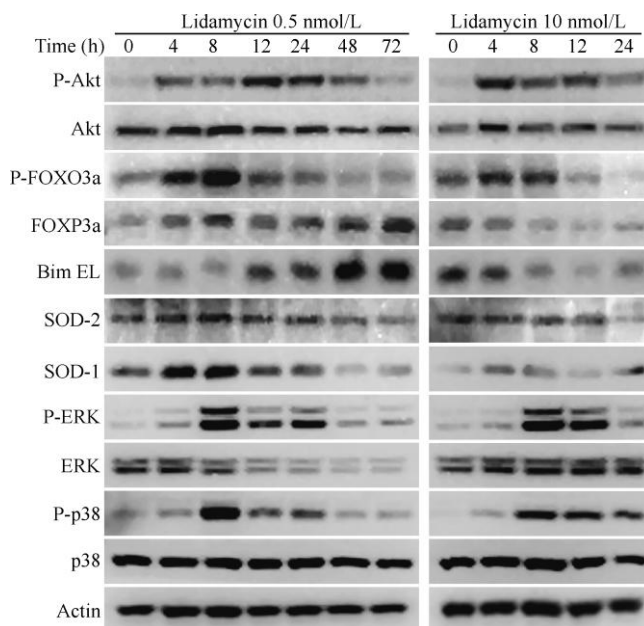

FIG. 2. Expression profiles of Akt/FOXO3a and MAPK signaling pathways after treatment of BEL-7402 cells with 0.5 nmol/L or 10 nmol/L lidamycin. The cell pellets were collected at indicated time points and analyzed by Western blot. The experiment was repeated twice and similar results were obtained.

Involvement of Akt and SIRT1 in Sensitivity to Lidamycin in HCT 116 p53$^{-/-}$ Cells

Our previous study showed that apoptosis is much more efficiently induced by lidamycin in HCT116 p53 wt cells than in HCT116 p53$^{-/-}$ cells[10], suggesting the involvement of prosurvival molecules. To test this notion, we evaluated the SIRT1 and Akt expression in both cell lines 24 h after lidamycin treatment. Compared with p53 wt cells, less PARP cleavage fragments were observed in p53$^{-/-}$ cells (Fig. 3), which is consistent with our previous results[10]. The total Akt and SIRT1 levels decreased in p53 wt cells 24 h following exposure to 10 nmol/L lidamycin, and increased in p53$^{-/-}$ cells after exposed to the same lidamycin concentration, indicating that prosurvival molecules such as SIRT1 and Akt are also associated with the action of lidamycin on this cell system.
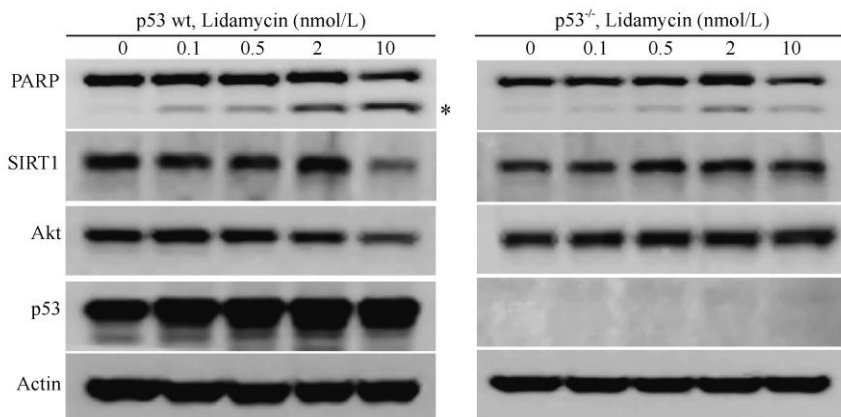

FIG. 3. Expression levels of Akt and SIRT1 and sensitivity to lidamycin-induced apoptosis in HCT 116 cells. The cells were treated with indicated lidamycin concentrations for 24 h. The protein levels were analyzed by Western blot. *: cleaved PARP fragments. The experiment was repeated twice and similar results were obtained.

Degradation of PARP and p53 Proteins at High Concentrations of Lidamycin

It was difficult to determine whether prosurvival molecules such as SIRT1 play a role in the mode of cell death as it results from inactivation of caspase during mitotic cell death. According to our hypothesis, distinct levels of specific proteins should be present after the cells are exposed to high concentrations of lidamycin. Degradation of PARP and p53, instead of SIRT1 and Akt, was observed 6 h after MCF-7 cells were treated with 0.1 μmol/L and 1 μmol/L lidamycin, respectively (Fig. 4A). Rapid degradation of PARP was also observed in BEL-7402 and HCT116 cells after incubation for 0.5 h with lidamycin (Fig. 4B), Suggesting that the selective degradation of apoptotic sensor proteins may be involved in the mode choice of cell death.

There are three pathways of protein degradation triggered by lidamycin stimuli: caspase activation, activation of proteasome pathway and direct degradation by lidamycin itself. We chose the general caspase inhibitor, Q-VD-OPh, to inhibit caspase activation as it is more efficient than the pan-caspase inhibitor Z-vad-fmk in DOX-triggered apoptosis[21]. The degradation of PARP and p53 was not influenced by addition of Q-VD-OPh (Fig. 4C). Less cleaved PARP fragments due to the activation of caspases were observed (Fig. 4C), which is consistent with our previous report that chromatin condensation induced by high concentration of lidamycin precedes the activation of caspases[6]. Concerning the proteasome pathway, we found that degradation of PARP triggered by lidamycin was not inhibited by addition of MG132 (50 μmol/L), a very efficient inhibitor of the proteasome pathway (Fig. 4D). The high p53 level after incubation with MG132 alone (Fig. 4D) is due to the inhibition of MDM2-mediated ubiquitination and subsequent proteasome degradation[22]. Compared with lower p53 level triggered by lidamycin alone, addition of MG132 partly inhibited the degradation of p53, indicating that the proteasome pathway is partly involved in lidamycin-triggered protein degradation.

Resistance to Lidamycin-induced DNA Cleavage in Doxorubicin-resistant MCF-7 Cells

To identify other cellular factors playing a role in

the action of lidamycin, we used an apoptosis-resistant MCF-7/DOX cell line for further experiments. First, we compared the cleavage in sensitive and resistant cells as rapid DNA cleavage at the linkage between nucleosomes has been observed in BEL-7402 and sensitive MCF-7 cells[6]. To our surprise, resistance to DNA cleavage in resistant MCF-7/DOX cells occurred 1 h after treatment with 1 μmol/L lidamycin (Fig. 5A). Additionally, chromatin condensation was observed. The chromatin condensed
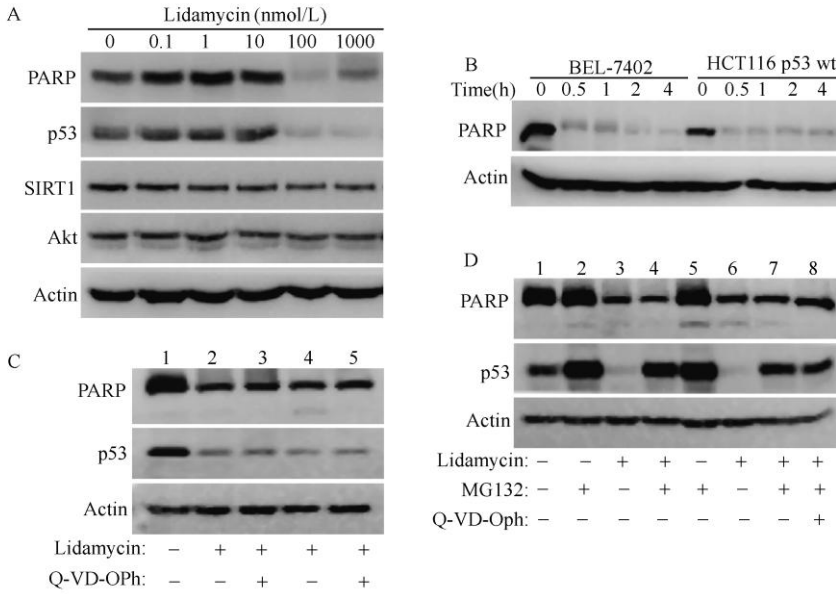

FIG. 4. Degradation of PARP and p53 proteins induced by high concentrations of lidamycin. A: Protein degradation patterns in MCF-7 cells triggered by various concentrations of lidamycin for 6 h. B: Rapid degradation of PARP by 1 μmol/L lidamycin incubation in BEL-7402 and HCT116 cells. C: No impact on degradation of PARP and p53 triggered by lidamycin with the general caspase inhibitor. MCF-7 cells were treated with 1 μmol/L lidamycin for 2 h (Lanes 2, 3), 6 h (Lanes 4, 5). Lanes 3, 5: plus 20 μmol/L caspase inhibitor Q-VD-OPh. D: Lidamycin-triggered protein degradation partly affected by the proteasome pathway. MCF-7 cells were treated with 1 μmol/L lidamycin for 2 h (Lanes 3, 4), 6 h (Lanes 6-8). Lanes 2, 4: Addition of 50 μmol/L MG132 for 3 h. Lanes 5, 7, 8: Addition of 50 μmol/L MG132 for 7 h. The inhibitors were added 1 h prior to lidamycin. This is a representative result from three independent experiments.
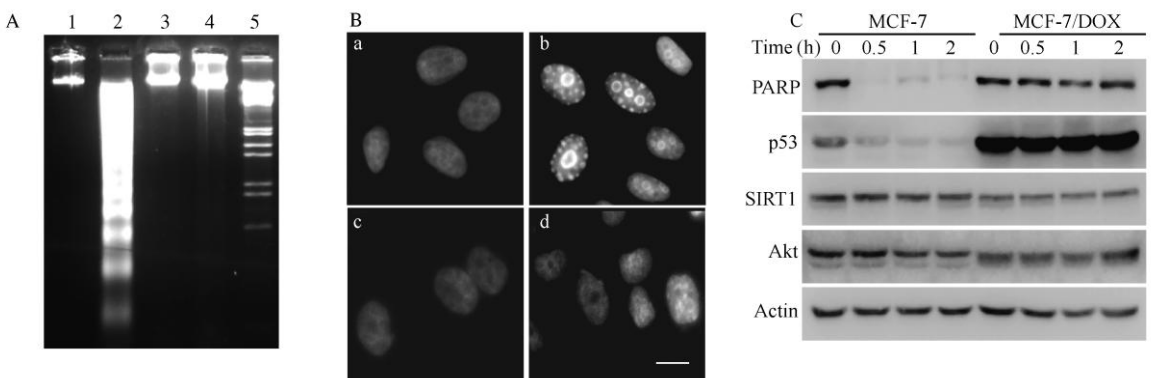

FIG. 5. Resistance to lidamycin-induced cleavage in doxorubicin-resistant MCF-7 cells. A: The DNA cleavage patterns 1 h after incubation with 1 μmol/L lidamycin. Lane 1: MCF-7 control cells; lane 2: lidamycin-treated MCF-7 cells; lane 3: MCF-7/DOX control cells; lane 4: lidamycin-treated MCF-7/DOX cells; lane 5: DNA markers. B: Chromatin condensation of MCF-7 (a: control cells; b: lidamyicn-treated cells) and MCF-7/DOX cells (c: control cells; d: lidamycin-treated cells) 1 h after incubation with 1 μmol/L lidamycin (bar in d: 40 μm). C: Degradation patterns of PARP, p53, SIRT1, and Akt proteins in sensitive and resistant MCF-7 cells 2 h after incubation with 1 μmol/L lidamycin. All the experiments were repeated at least three times.

to a "dot" type in sensitive MCF-7 cells 1 h after treatment with 1 μmol/L lidamycin for (Fig. 5B, a, b). In contrast, no chromatin condensation was observed under the same condition in the drug-resistant cells (Fig. 5B, c, d). MCF-7/DOX cells were also resistant to protein degradation triggered by lidamycin. In contrast to the rapid degradation of PARP and p53 proteins in MCF-7 cells, their levels remained constant 2 h after lidamycin (1 μmol/L) incubation (Fig. 5C).

We also detected the growth-inhibitory effect of lidamycin on both cell lines by the MTT method, showing weak resistance in MCF-7/DOX cells (data not shown). We compared the difference of mitotic cell death in two cell lines at lower lidamycin concentrations. As shown in Fig. 6A, the percentage of cells with senescence-associated β-galactosidase (SA-β-gal) expression was similar in MCF-7/DOX and MCF-7 cells 72 h after exposure to lidamycin, depending on lidamycin concentrations. However, the high percentage of multinucleation, one of the main features of mitotic cell death, required a higher concentration of lidamycin in MCF-7/DOX cells (Fig. 6B). The peak was reached in MCF-7 cells and

MCF-7/DOX cells, respectively, after treatment with 0.1 nmol/L and 0.5 nmol/L lidamycin, while the large reduction of multiunucleation at high concentrations of lidamycin was due to apoptosis, suggesting that there are cellular factors that can delay the action of lidamycin on MCF-7/DOX cells.

Lidamycin is not a Substrate of P-glycoprotein (P-gp)

To rule out the possibility that lidamycin is transported out by P-gp, we used flow cytometry to detect the accumulation of rhodamine 123, a substrate of P-gp, in MCF-7/DOX cells following treatment with lidamycin. Compared with a lower fluorescent intensity in MCF-7/DOX cells, a large increase of rhodamine 123 accumulation was observed after incubation with 2 μmol/L tetrandrine, a very efficient reverser of P-glycoprotein-mediated drug resistance (Fig. 7A, Line 3)[23]. However, rhodamine 123 accumulation was not increased in MCF-7/DOX cells after incubation with 2 nmol/L and 10 nmol/L lidamycin, respectively (Fig. 7B).
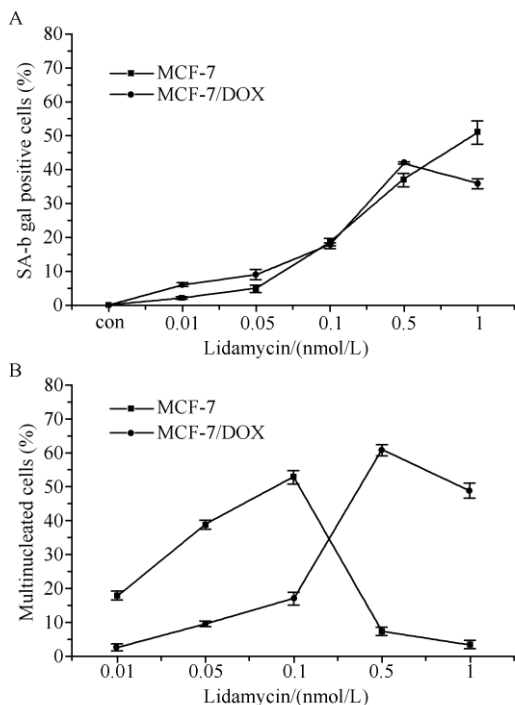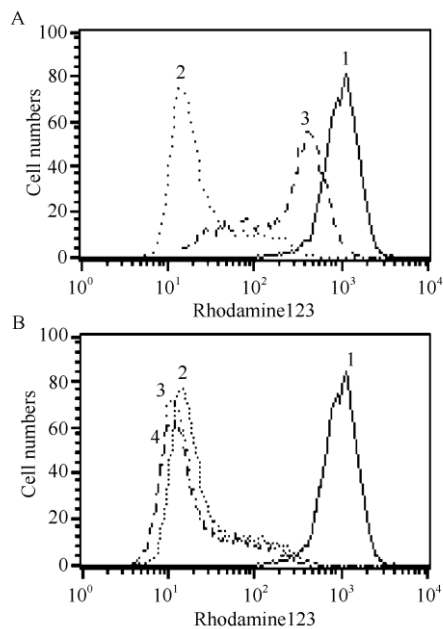

FIG. 7. No accumulation of rhodamine 123 in MCF-7/DOX cells detected by flow cytometer after exposure to lidamycin. A: Tetrandrine greatly increased rhodamine 123 accumulation. Line 1: sensitive MCF-7 cells; line 2: MCF-7/DOX cells; line 3: MCF-7/DOX cells 1 h after treatment with 2 μmol/L tetrandrine; B: No increase of rhodamine 123 accumulation following incubation with lidamycin. Line 1: sensitive MCF-7 cells; line 2: MCF-7/DOX cells; line 3: MCF-7/DOX cells 1 h after treatment with 2 nmol/L lidamyicn; line 4: MCF-7/DOX cells 1 h after treatment with 10 nmol/L lidamycin. This is a representative result from three separate experiments.

FIG. 6. Percentages of mitotic cell death in MCF-7 and MCF-7/DOX cells 72 h after exposed to lidamycin. A: The percentages of cells with SA-β-gal expression; B: The percentages of multinucleated cells. Data were means of three separate experiments.

DISCUSSION

In the present study, we found that the prosurvival molecules such as SIRT1, Akt, and SODs could impact the action of lidamycin on tumor cells, which is an important milestone on the way to understand the mechanism by which lidamycin exerts its potent action on tumor cells, and to the mechanism of mitotic cell death or mitotic catastrophe. There is ample evidence that in addition to apoptosis, other types of cell death, such as mitotic cell death, autophagy and senescence also play an important role in tumor chemotherapy[24-25]. Sometimes, an antitumor agent can induce two types of cell death. The characteristics of cell death induced by lidamycin, as a DNA damaging agent, are similar to thathose of DOX[13]. Transient p38 phosphorylation at lower lidamycin concentrations and maintenance of p38 phosphorylation in apoptosis are also observed in DOX-triggered cell deaths[26].

SIRT1 deacetylase participates in a number of physiological processes, for instance, glucose homeostasis, fat mobilization, response to calorie restriction and anti-aging[17,27-28], and can promote cell survival *via* deacetylated p53 in DNA damage response[29]. SIRT1 protein is highly expressed in tumor cells. In addition to suppression of apoptosis, SIRT1 directly regulates *mdr-1* gene expression, which codes P-gp to confer multidrug resistance on tumor cells[30]. Interestingly, p53, FOXO3a, and SIRT1 form a feedback loop to modulate SIRT1 expression[31]. In HCT116 p53 knock-out cells, the present results show an increased SIRT1 and Akt expression in response to lidamycin stimuli, implying the involvement of dysregulation of p53 in mitotic cell death. In cisplatin induced cell death, apoptosis requires p53 and caspase-2, and a deficiency of p53 leads to mitotic cell death[32]. Using HCT116 p53 deficient cells, we are currently investigating the regulation of SIRT1 expression through siRNA and transferring SIRT1 gene to modulate cell death.

In this study, lidamycin degraded specific proteins instead of prosurvival proteins, such as SIRT1 and Akt within the cells (Fig. 4). In support of these findings, lidamycin has an aminopeptidase activity *in vitro* that could not be inhibited by the protease inhibitor, leupeptin[33]. The degradation of histone H1 by lidamycin *in vitro* has been described elsewhere[6]. The selective interaction of lidamycin with proteases may be a reflection of its complex toxicity to tumor cells, which may also be involved in the mode of cell death. The question why some proteins are degraded by lidamycin but not by SIRT1 remians to be answered.

The mechanism by which MCF-7/DOX cells are resistant to lidamycin-induced cleavage remains elusive. There are several explanations for this phenomenon. Firstly, the possibility that lidamycin is a substrate of P-glycoprotein was ruled out (Fig. 7). Secondly, glutathione in resistant cells might have been bound to the chromophore of lidamycin. However, MCF-7/DOX cells 24 h after pretreated with glutathione synthesis inhibitor, L-buthionine-sulfoximine, remained resistant to lidamycin-induced DNA cleavage (data not shown). Thirdly, the levels of prosurvival proteins SIRT1, SOD-1, and SOD-2 remained unchanged in MCF-7/DOX cells (data not shown). Lastly, altered nuclear structure might have delayed the entry of lidamycin into nuclei. It was reported that more pores exist in the nuclear membrane of multidrug resistant bladder cancer cells[34].

In summary, lidamycin exerts its potent cytotoxicity to human cancer cells by several mechanisms. The types of cell death depend on the balance between pro-apoptotic molecules and prosurvival molecules. A better understanding of the mechanism of cell death triggered by lidamycin, including the role of prosurvival molecules, can improve the chemotherapeutic efficacy of lidamycin and reduce its side effect.

ACKNOWLEDGEMENTS

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