RESEARCH ARTICLE

Over-Expression of Beclin-1 Facilitates Acquired Resistance to Histone Deacetylase Inhibitor-Induced Apoptosis

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Abstract

Apoptotic cell death plays a predominant role in histone deacetylase (HDAC) inhibitor-induced cytotoxicity. Nuclear morphological changes and activation of apoptotic executors are involved in CTS203-induced cell death. However, emerging issues of HDAC inhibitor-resistance have been observed in patients. Herein, MCF-7 cells were continuously exposed to CTS203 until the derived cells could proliferate normally in its presence. The newly obtained CTS203-resistant cells were nominated as MCF-7/203R. Compared to MCF-7 original cells, the MCF-7/203R cells were less sensitive to CTS203-induced apoptosis, with a minimal 6-fold higher IC_{50} value. In contrast, the expression of Beclin-1 was dramatically up-regulated, positively correlated to the acquisition of CTS203-resistance. Our results revealed the participation of autophagy in acquired HDAC inhibitor-resistance and further identified Beclin-1 as a promising target for anti-drug resistance.

Keywords: HDAC inhibitor - drug resistance - Beclin-1 - autophagy - apoptosis

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Introduction

Apoptosis is a well-known programmed cell death mechanism and plays a critical role in chemo stimuli-induced cytotoxicity (Debatin, 2004). Alternatively, autophagy which allows the degradation and recycling of cellular components to obtain energy would be triggered during chemo treatment. Unlike apoptosis, autophagy may otherwise facilitate cell survival against specified stresses depending on cellular context (Yang et al., 2011). Emerging evidence reveals that the crosstalk between apoptosis and autophagy is crucial in determination of cell fate (Eisenberg-Lerner et al., 2009). Therefore, appropriate treatments should be applied to manipulate the interplay between apoptosis and autophagy in order to enhance therapeutic efficiency. On one hand, the inhibition of cyto-protective autophagy would promote apoptotic cell death (Abedin et al., 2007). On the other hand, induction of autophagy would be lethal, especially for apoptosis-deficient cells (Carew et al., 2007; Sun et al., 2011).

Global gene profiling reveals that tumor malignancy usually correlates to abnormal expression of Beclin-1, such as partial deficiency in breast cancer cells or over-expression in epithelial ovarian cancer cells (Li et al., 2010; Cai et al., 2014). Therefore, Beclin-1 becomes an ideal target for anti-cancer treatment due to its correlation with tumor. Accordingly, the addition of autophagy inhibitor promoted the anti-cancer efficiency of radiotherapy (Apel et al., 2008). Furthermore, Beclin-1 could regulate the crosstalk between apoptosis and autophagy by directly interaction with Bcl-2 via its BH3 domain as well as by caspase-induced inactivation (Kang et al., 2011).

HDAC inhibitors are a class of promising anti-cancer agents. To date, there are dozens of HDAC inhibitors under clinical trials, individually or synergistically (Kim et al., 2011). Unfortunately, clinical outcomes revealed an emerging problem of acquired resistance to HDAC inhibitors (Juengel et al., 2013). However, the knowledge of the mechanism underlying acquired resistance is still quite limited. In our previous study, a novel HDAC inhibitor CTS203 exhibited more potential than its precursor compound TSA and thus was identified as a promising anti-cancer candidate (Wang et al., 2014). Herein, by continuous exposure to CTS203, a group of CTS203-resistant descendents was derived from MCF-7 cells and was nominated as MCF-7/203R. By investigation of the differences in cellular characterization between CTS203-resistant and CTS203-sensitive MCF-7 cells, our results indicated that autophagy was associated with the acquisition of CTS203-resistance as well as shed lights on the role of Beclin-1 in drug resistance.

Materials and Methods

Compounds and reagents

CTS203 (Figure 1) was prepared as previously described (Wang et al., 2014). 3-Methyladenine (3-MA) was purchased from Acros Organics (NJ, USA). CTS203 was dissolved in DMSO and was diluted to appropriate concentrations using normal culture medium. The final

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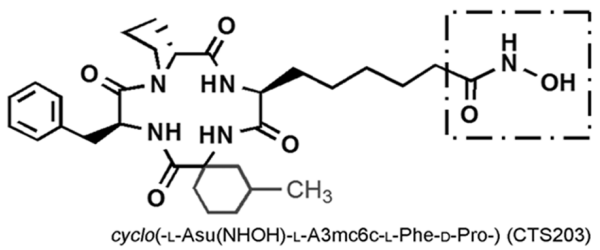

Figure 1. Structure of CTS203

concentration of DMSO was less than 0.1% (v/v). 3-MA was dissolved in PBS.

Cell culture

Malignant MCF-7 and HL60 cell lines were obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, China). MCF-7/203R cells were derived from MCF-7 cells by continuous treatment with CTS203. All the cells were maintained in RPMI 1640 medium supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C. The growth medium was routinely changed every 24h.

Cell viability measurement

Cell viability was evaluated via MTT (Sigma Aldrich, USA) assay. Cells were seeded in 96-well plates at a density of 5×10^3 per well, exposed to various concentrations of CTS203 for 48h. Cells exposed to DMSO under the same conditions were used as vehicle control. After the indicated treatment, MTT was added and the mixture was maintained for another 3h. After reaction, the supernatant was aspirated and the blue formazan precipitates were dissolved in DMSO. After gentle agitation, the absorbance (A) was ultimately measured on a multi-well reader (Thermo Fisher Scientific, USA). The inhibition ratio was calculated as follow:

$$\text{Inhibition Ration (\%)} = (1 - (A_{570treated} - A_{630treated}) / (A_{570treated} - A_{630treated})) \times 100\%$$

Cell cycle and apoptosis analysis

Cell cycle distribution and the induction of apoptosis were determined via propidium iodide (PI, Sigma Aldrich, USA) staining. Briefly, cells were seeded in 6-well plates and treated with indicated concentrations of CTS203 for 24 h. After incubation, cells were harvested and fixed with 75% cold ethanol overnight. The fixed cells were washed twice with cold PBS, subsequently re-suspended in PI solution containing 10mg/mL RNase and 1% Triton X-100 and incubated for 20 min in darkness. The stained cells were finally measured using a flow cytometry system (BD, USA) and the results were analyzed using Mod Fix LT3.0 software.

Fluorescence microscopy

Morphological features of apoptosis were determined by Hoechst 33342 staining. Briefly, cells grown on cover slips were treated with CTS203 for 24h. After the indicated treatment, cells were washed with PBS and then fixed with 4% paraformaldehyde (PA, AMERSCO, USA) for 90s. After disposure of PA, the fixed cells were stained with Hoechst 33342 (AMERESCO, USA) for 20 min in darkness at room temperature. After rinse with PBS, the stained cells were ultimately examined under a fluorescence microscope (Olympus 1X71, Japan).

Immunofluorescence assay

The appearance of autophagosome was determined by immunofluorescence assay. Cells grown on cover slips were fixed in 4% cold PA for 20 min at room temperature, followed by permeabilization in PBS-T solution (0.1% Triton X-100 in PBS) for 10 min at 4°C. Subsequently, the permeabilized cells were blocked with PBS-B solution (4% BSA (Thermo Fisher Scientific, USA) in PBS) for 30 min at 37°C. Then the blocked cells were incubated with primary anti-LC3 antibody (1:100, Abcam, UK) at 4 °C overnight for probing, followed by incubation with FITC-labeled secondary antibody (1:200, Thermo, USA) for 1 h at room temperature. After rinse with PBS, cells were ultimately examined under a confocal microscope (Olympus FV1000, Japan).

Western blot

The expression levels of target proteins were detected by western blot. To standardize protein input, β-actin was used for normalization.

Whole cell extracts were prepared and individual protein concentrations were quantified using the BCA protein assay kit (KeyGen, China). Equal amount of protein was loaded on each lane, separated on a 15% SDS-PAGE gel and then transferred onto a PVDF membrane (Millipore, USA). The membrane was saturated with 5% *(w/v)* skim milk (dissolved in TBS containing 0.1% Tween-20) overnight at 4°C, followed by incubation with specific primary antibodies against Beclin-1 (1:800, Proteintech, USA) or β-actin (1:1500, Santa Cruz, USA) overnight at 4°C. After rinses, the membrane was incubated with HRP-conjugated secondary antibody (1:2000, Santa Cruz, USA) for 1 h at 37°C. The corresponding protein bands were visualized using an ECL reagent kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

To determine the release of cytochrome c from mitochondria, cytoplasmic extracts were prepared after the indicated treatment. A cytochrome c detection kit (KeyGen, China) was specially used and all follow-up procedures were performed strictly according to the manufacturer's instructions.

Statistical analysis

All data represent at least 3 independent experiments and are expressed as Average±SD unless otherwise indicated. To evaluate differences between control and treatment groups, a paired Student's *t*-test was performed. ***p*-value less than 0.01 was considered statistically extremely significant.

Results

Apoptotic cell death induced by CTS203

Once apoptosis is triggered, morphological changes such as apoptotic bodies and the irregular shaped nucleus would appear. As shown in Figure 2 and Figure 3, after

exposure to CTS203, both MCF-7 cells and HL60 cells were morphologically changed, accompany with deeply stained but irregularly shaped nucleus. In HL60 cells, unevenly stained dots could be clearly spotted in the enlarged pictures.

In addition, the release of cytochrome c from mitochondria and the activation of caspase-3 further confirmed the occurrence of apoptosis. The activity of caspase-3 is closely related to the execution of apoptotic program. Similarly, cytoplasmic accumulation of cytochrome c is also an important step in the initiation of apoptotic cascade. Given MCF-7 cells are genetically deficient for caspase-3 (Janicke et al., 2009), the variation of cytochrome c was then analyzed to determine the status of apoptosis in this specific cell line. Just like the results obtained from nucleus staining, the cytoplasmic accumulation of cytochrome c in MCF-7 cells (Figure 2) and the activity of caspase-3 in HL60 cells (Figure 3) were both up-regulated due to the presence of CTS203, with at least 4-fold or 40-fold increase, respectively.

Interestingly, the activity of caspase-3 in MCF-7 cells yet slightly up-regulated after exposure to CTS203. Such variation might be arouse by its isoenzymes caspase-6/7 which are occasionally responsible for executing apoptosis, especially in caspase-3-deficient cells (Janicke et al., 2009).

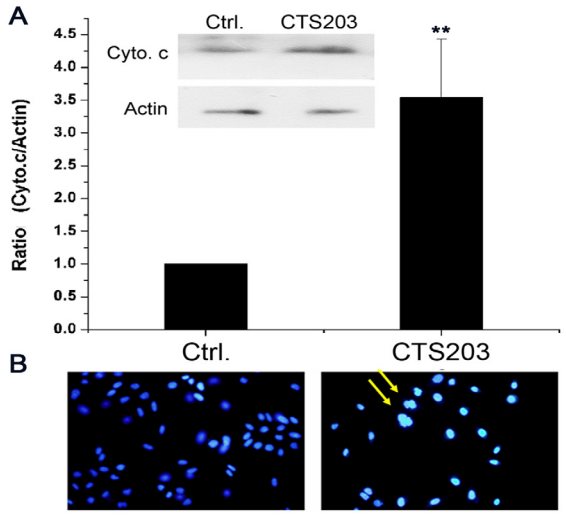

Figure 2. Induction of Apoptotic Cell Death in MCF-7 Cells. A: Effect of CTS203 on the release of Cytochrome c. Cytoplasmic extracts were prepared after the indicated treatment for 24h. Equal loading and transfer were verified by probing the membranes with anti-Actin antibody. **B:** Hoechst 33342 staining (magnification: ×200). Cells were stained with Hoechst 33342 after the inficated treatment for 24h

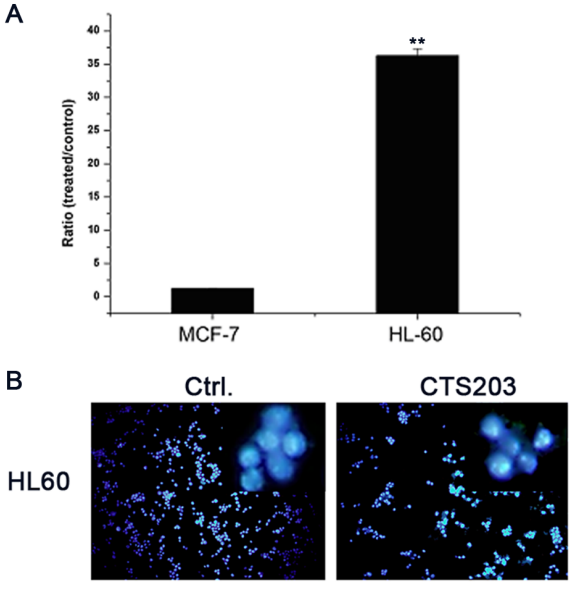

Figure 3. Induction of Apoptotic Cell Death in HL60 Cells. A: Effect of CTS203 on the activation of caspase-3. Whole cell Extracts were prepared after the indicated treatment for 24h. **B:** Hoechst 33342 staining (magnification: ×200). Cells were stained with Hoechst 33342 after the inficated treatment for 24h. Each insert was an enlarged image

The induction of CTS203-resistance in MCF-7 cells

To obtain CTS203-insensitive cells, MCF-7 cells were continuously exposed to CTS203 until their descendents can normally grow and proliferate in the presence of CTS203. The derived cells were nominated as MCF-7/203R. The proliferative characterization of MCF-7/203R cells was then analyzed so as to determine their CTS203-resistance index. As shown in Figure 4, the cell viabilities of MCF-7 and MCF-7/203R cells were both inhibited by CTS203, appearing a similar concentration-dependent manner. However, the IC_{50} value of CTS203 against MCF-7/203R cells (160 nM) were significantly increased as compared to that of original MCF-7 cells (26 nM), exhibiting a minimal 6-fold decreased susceptibility to CTS203.

MCF-7/203R cells are less sensitive to CTS203-induced apoptotic cell death

To determine whether apoptosis-inducing ability would be affected by the acquisition of CTS203-resistance, apoptotic proportion and cell cycle distribution were then analyzed by flow cytometry. As shown in Figure 5, both CTS203 resistant and sensitive cells were mainly blocked in G_0/G_1 and Sub G_1 phases, indicating cell cycle distribution of both MCF-7 cells and MCF-7/203R cells were affected by CTS203. Specifically, the

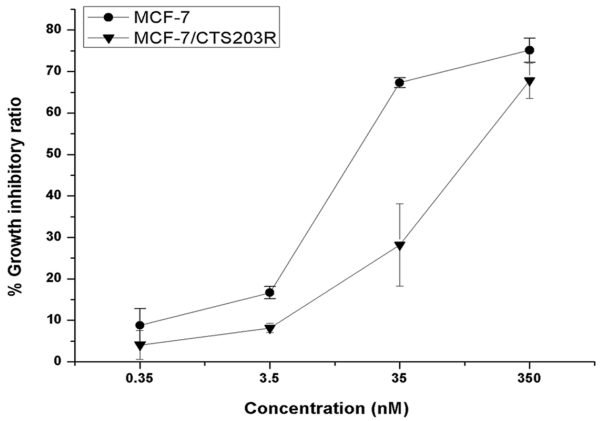

Figure 4. Susceptibility of MCF-7/203R Cells to CTS203. Cells were treated with indicated concentration of CTS203 for 48h and then subjected to cell viability measurement

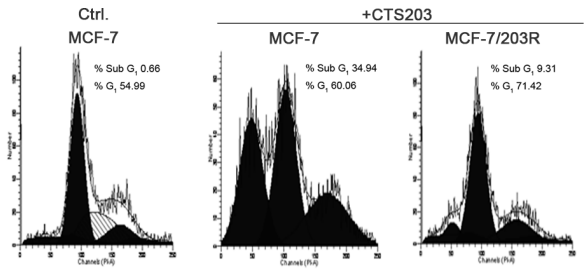

Figure 5. Effects of CTS203 on Cell Cycle Distribution and Apoptosis. Cells were harvested after the indicated treatment for 24h and then subjected to flow cytometry analysis

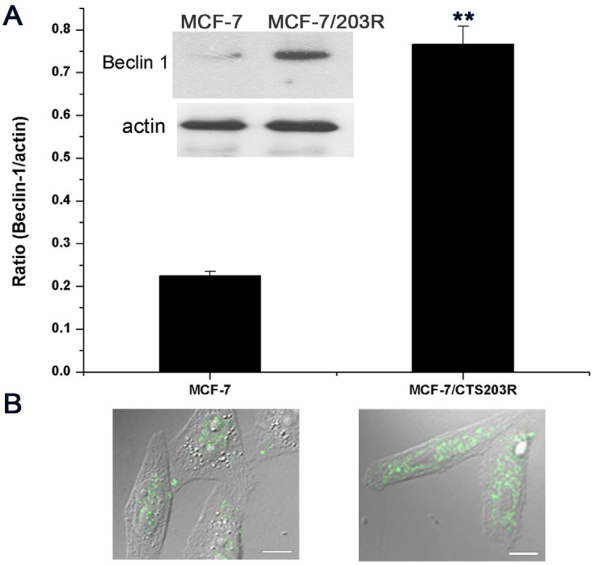

Figure 6. Basal Level of Autophagy in CTS203-Resistant and CTS203-Sensitive MCF-7 Cells. A: The accumulation of Beclin-1 in MCF-7/203R cells. Whole cell extracts were prepared and then subjected to western blot. Equal loading and transfer were verified by probing the membranes with anti-Actin antibody. **B:** The increase of autophagic vacuoles. Cells were probed with primary anti-LC3 antibody, subsequently incubated with FITC-labeled secondary antibody before observation. Scale Bar: 10 μm

percentage of MCF-7 cells accumulated in Sub G_1 phase was dramatically increased from less than 1% to as high as 35% after treatment, while the percentage of cells arrested in G_1 phase was gently increased from 55% to 60%. In contrast, the percentage of MCF-7/203R cells accumulated in Sub G_1 phase was gently increased to 9% after treatment, while the percentage of cells blocked in G_1 phase dramatically increased to 71%. Collectively, in comparison to the significant pro-apoptotic effect of CTS203 on MCF-7 cells, the cytotoxic effect of CTS203 on MCF-7/203R cells was merely cell cycle arrest.

Beclin-1 was over-expressed in MCF-7/203R cells

Besides facilitating cell survival against chemo-stresses, accumulating evidence also implied the involvement of autophagy in acquired drug resistance (Yokoyama et al., 2009). To determine the participation of autophagy in the acquisition of CTS203-resistance, autophagic parameters were then analyzed.

LC3 is a specific marker of autophagy; its cellular amount positively correlates to the occurrence of autophagy. As shown in Figure 6, more fluorescent spots which represents an autophagosome each appeared in MCF-7/203R cells as compared to the original MCF-7 cells, indicating the basal level of autophagy was permanently up-regulated.

In addition, the expression of Beclin-1 further confirmed the promotion of basal autophagy. Consistent with the increased amount of autophagosomes, the accumulation of Beclin-1 was dramatically increased in MCF-7/203R cells as compared to the original MCF-7 cells. Furthermore, the densitometric analysis of western blot indicated a minimal 3-fold higher accumulation of Beclin-1 in MCF-7/203R cells as compared to MCF-7 cells, suggesting the positive correlation between Beclin-1 and acquired CTS203-resistance.

Discussion

HDAC inhibitors are a novel class of promising anti-cancer agents. By introducing proper modifications into the original scaffolds of classical HDAC inhibitors, more efficient derivatives would be obtained. In our previous study, a novel HDAC inhibitor CTS203 was screened out, exhibiting excellent HDAC inhibitory activity and significant antiproliferative activity. Like its precursor compound TSA, apoptotic cell death could be triggered by CTS203 (Figure 2, Figure 3).

Cell cycle arrest and apoptosis are two major antiproliferative mechanisms which are generally induced by HDAC inhibitors. Likewise, CTS203 induced cell cycle arrest and subsequent apoptosis in MCF-7 cells (Figure 5). It has been reported that the decision between cell cycle arrest and apoptosis is concentration-dependent (Kim et al., 2011). Similarly, when MCF-7 cells were exposed to CTS203 at a concentration of 100 nM, which is higher than its IC_{50} value, more than one third of MCF-7 cells underwent apoptosis. In contrast, few MCF-7/203R cells ended up undergoing apoptosis under the same conditions, whereas most cells were mainly blocked in G_1 phase due to the increased IC_{50} value (160 nM) of CTS203. Namely, the lowered sensitivity to apoptosis led to relative weak cytotoxicity. Given such resistance to apoptosis is quite similar to the pro-survival effect driven by autophagy (Fantin et al., 2010), we further investigated the role of autophagy in acquisition of CTS203-resistance.

Increasing evidence indicates that autophagy is probably involved in acquired drug resistance (Yokomaya et al., 2009). Likewise, our results argued that the basal level of autophagy was enhanced in CTS203-resistant cells (Figure 6), implying the participation of autophagy in the acquisition of CTS203 resistance. Furthermore, the expression of Beclin-1, a necessary initiator to activate autophagy, was further accumulated in MCF-7/203R cells, appearing a positive correlation to CTS203-resistance. According to the recently reported role of autophagy in degradation of active caspase-8 (Hou et al., 2010), the promotion of basal autophagy therefore lowered the sensitivity of MCF-7/203R cells to apoptosis (Figure 5), consequently led to decreased CTS203-induced cytotoxicity (Figure 4).

Many researches have been conducted to investigate

the underlying mechanism of acquired chemo-resistance. As a result, mediators including MDR1, *p*-glycoprotein, Bcl-xl and estrogen receptor α have been identified as resistance-related factors, which are abnormally expressed in different types of chemo-resistant cell lines (Chen at al., 2012; Xu et al., 2013). But evidence from numerous researches alternatively argued that acquired resistances aroused by distinct stimuli would be ascribed to different executors, such as caveolin-1 in Taxol-resistant ovarian carcinoma cells, Ki-67 in paclitaxel-resistant breast cancer cells and E-cadherin in 5-FU-resistant hepatocellular carcinoma cells (Gu et al., 2012; Chen et al., 2013; Wang et al., 2013). Therefore, specific regulators associated to the formation of drug resistance are still waiting to be discovered, especially the cell type-dependent ones. Herein, our data indicated the involvement of Beclin-1 to acquired HDAC inhibitor-resistance in MCF-7 breast cancer cells.

In summary, CTS203-resistant MCF-7 cells were successively derived and showed a resistance index of 6. Beclin-1 was up-regulated in CTS203-resistant MCF-7 cells, thus lead to apoptosis deficiency. Therefore, Beclin-1 would be a promising anti-resistance target during prolonged anti-cancer treatment.

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