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3-Methyladenine induces cell death and its interaction with chemotherapeutic drugs is independent of autophagy

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ABSTRACT

3-Methyladenine (3-MA) is an autophagy inhibitor and has been widely used as a pharmacological tool in the autophagy studies. 3-MA potentiates the chemotherapeutic effects of anticancer drugs, but it is not clear whether the potentiating effects of 3-MA on chemotherapy efficacy comes from the autophagy inhibition or not. The aim of the present work is to identify the relationship between the effects of 3-MA on chemotherapy and the 3-MA-induced autophagy inhibition. The autophagy responses were evaluated by measuring LC3-II level. Cell viability, cell death and cell apoptosis were evaluated by MTT, live and dead assay kit and Tunel staining. Results showed that 3-MA dose-dependently reduced Hela cell viability but did not affect the basal autophagy responses. 3-MA at the concentration that inhibits autophagy induced Hela cell death and apoptosis. 3-MA did not inhibit the increased autophagy responses induced by chemotherapeutic drugs cispcis-diamminedichloroplatinum(II) (CDDP), tamoxifen and 5-fluorouracil (5-FU) in Hela and MCF-7 cells. The synergism or antagonism between 3-MA and chemotherapeutic drugs was dependent on the inhibition ratio of tumor cells. In conclusion, 3-MA itself induces cell death and apoptosis without relationship with autophagy; 3-MA does not inhibit the increased autophagy induced by anti-cancer drugs; the interaction between 3-MA and chemotherapeutic drugs is not related to autophagy.

1. Introduction

Autophagy is an evolutionally catabolic process involving the degradation and turnover of cytoplasmic material in lysosomes through the lysosomal machinery. The basal autophagy is to remove the malfunctioning organelles or damaged proteins, and maintains cellular homeostasis and genomic integrity, but when it is extensive, autophagy acts as an alternative cell death pathway [1]. Autophagy has been linked with variety of diseases including cancer [2], cardiovascular diseases [3] and neurodegenerative disorders [4].

Numerous evidences suggest that autophagy is a survival mechanism that provides energy during metabolic stress and protects cancer cells from apoptotic or necrotic cell death induced by various anticancer treatments [5]. Therefore, inhibiting autophagy can enhance the efficacy of anticancer therapies [6]. Several studies have reported that inhibition of autophagy sensitizes cancer cells to chemotherapy and gene therapy [6–8]. 3-Methyladenine (3-MA) is identified as an autophagy inhibitor since 1982 [9] and has been widely used as a pharmacological tool in the studies of autophagy [4,10–12]. However, recent works showed that 3-MA was not a specific autophagy inhibitor, it also inhibited PI3K [13] , stimulated cAMP-dependent protein kinase (PKA) [14]. Thus, it needs to be addressed whether the potentiating effects of 3-MA on chemotherapy efficacy comes from the autophagy inhibition or not. The aim of the present work is to identify the relationship between the effects of 3-MA on chemotherapy and the 3-MA-induced autophagy inhibition.

2. Materials and methods

2.1. Agents

3-Methyladenine (3-MA), cispcis-diamminedichloroplatinum (II) (CDDP), 5-fluorouracil (5-FU) and anti-LC_3B were purchased from Sigma–Aldrich. Tamoxifen was from Tocris Bioscience. The medium was Hyclone product. Anti-actin was purchased from Cell Signaling Technology.

2.2. Cell culture

MCF-7 breast cancer cells and Hela cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin

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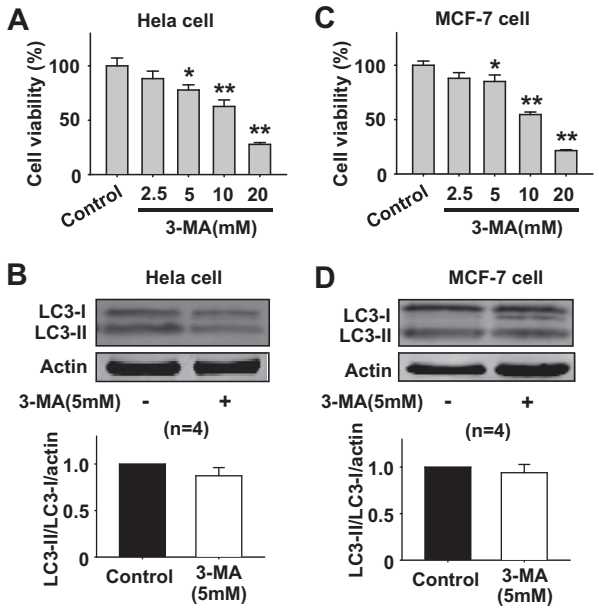

Fig. 1. 3-MA reduced cell viability without changing the basal autophagy. (A) 3-MA dose-dependently reduced Hela cell viability after 24 h treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. $n = 6$. (B) 3-MA(5 mM) did not affect the basal autophagy after 24 h treatment in Hela cells. Autophagy was evaluated by measurement of LC3-II protein level. $n = 4$. (C) 3-MA dose-dependently reduced MCF-7 cell viability after 24 h treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. $n = 6$. (D) 3-MA(5 mM) did not affect the basal autophagy after 24 h treatment in MCF-7 cells. Autophagy was evaluated by measurement of LC3-II protein level. $n = 4$.

at 37 °C, 5%CO_2. The time of treatment and the concentration of agents were shown in figures and/or figure legends.

2.3. Western blot analysis

Western blot test was performed as shown in our previous works [15]. Cells were lysed with RIPA buffer containing 1% protease inhibitor and centrifuged at 15,000*g* for 15 min at 4 °C. The supernatants were collected, and the protein concentrations were determined with BCA Protein Assay Kit (Bio-Rad). The proteins were electrophoresed in 10–15% SDS–PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk in phosphate buffered saline (PBS) for 2 h at room temperature, the membranes were incubated with the primary antibodies against LC_3B (1:2000), actin (1:1000) at 4 °C over night. After washing with PBS-0.1% Tween 20 (PBST), membranes were incubated with fluorescence-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody (Invitrogen, 1:10,000) at room temperature for 1 h. The band densities were quantified by densitometry using Odyssey v3.0 software.

2.4. TUNEL staining

After 3 times PBS washing, treated cells were fixed by 4% paraformaldehyde, permeabilized in 0.1% Triton x-100 sodium citrate buffer. Then an in situ cell death detection kits (Roche) were used to label apoptotic cells, and the nuclei were stained with DAPI. The number of total cells and tunel positive cells were automatically counted by Image-Pro plus version. The apoptosis rate was defined as ratio of apoptotic cells to total cells.

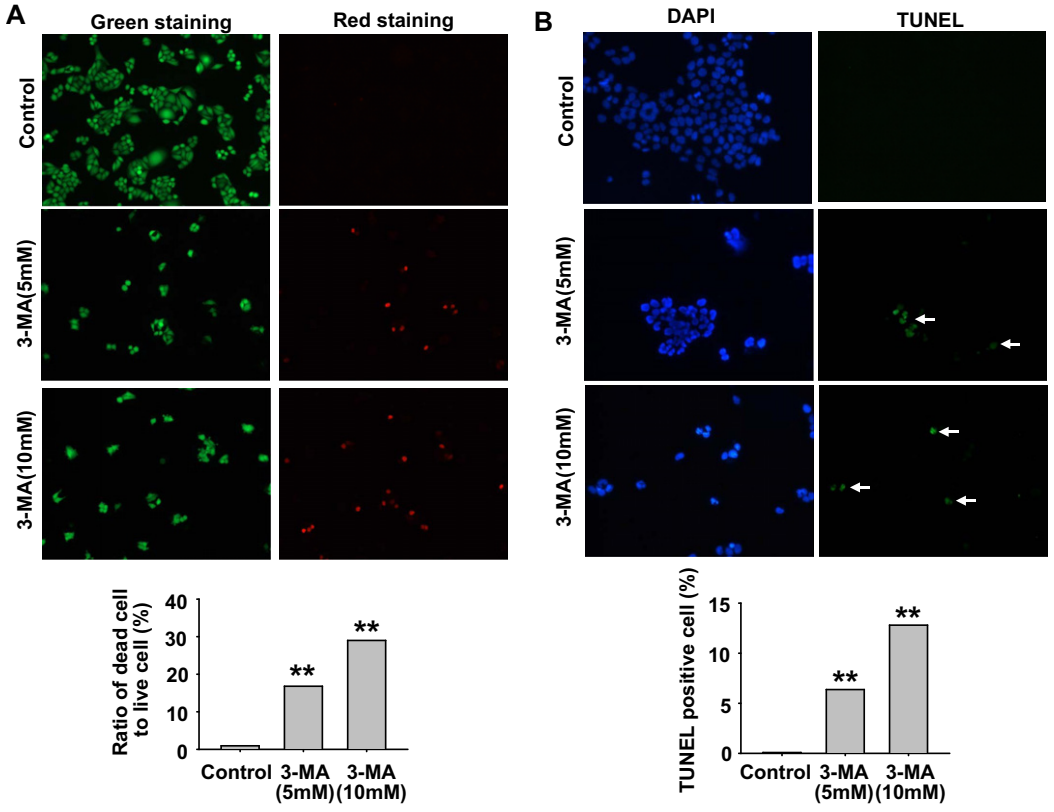

Fig. 2. 3-MA induced cell death and apoptosis. (A) Live and dead assay showed that 3-MA induced Hela cell death. Cells were treated with 3-MA for 24 h. Live cells were stained green and dead cells were stained red. The analyzed number of total green cells in control, 5 mM 3-MA, and 10 mM 3-MA groups was 1815, 761, and 713 respectively. χ^2-test was used, $^{**}P < 0.01$, vs. control. (B) TUNEL staining showed that 3-MA induced Hela cell apoptosis. The apoptosis cells were stained green. Cells were treated with 3-MA for 24 h. The analyzed number of total cells in control, 5 mM 3-MA, and 10 mM 3-MA groups was 1203, 674, and 1259 respectively. χ^2-test was used, $^{**}P < 0.01$, vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. MTT assay

Viability of cells cultured in the 96-well culture plates was assessed by measuring mitochondrial dehydrogenase activity, using the colorimetric MTT assay, as shown in our previous works [15].

2.6. Live- and dead-cell staining

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen) was used to detect live and dead cells [15]. The labeled cells were photographed under a fluorescence microscope. The live cells fluoresce green and dead cells fluoresce red.

2.7. Evaluation of interaction between 3-MA and CDDP in Hela cells

In order to identify the summation, synergism and antagonism interaction between autophagy inhibitor 3-MA and anti-cancer drugs CDDP and tamoxifen in Hela and MCF-7 cells, we used 3-MA with CDDP and tamoxifen at a fixed ratio (3-MA: CDDP/tamoxifen = 750:1 (μM)) to inhibit the proliferation of Hela and MCF-7 cells. The maximal concentrations of 3-MA and CDDP/tamoxifen at the fixed ratio were 3-MA (15 mM) and CDDP/tamoxifen (20 μM), and the concentrations were decreased at the decrement ratio of 0.75, 0.5, 0.25, and 0.1. After treatment with the combination of 3-MA and CDDP/tamoxifen for 24 h, the cell viability was examined with MTT method and the data were subsequently analyzed by the median effect methods developed by Chou and Talalay [16,17]. Combination index (CI) was calculated at the fraction ranged from 0.2 to 0.8 with the assumption that 3-MA and CDDP/tamoxifen were either mutually exclusive ($a = 0$) or non-exclusive ($a = 1$). CI value represented the interaction effects of the drugs. CI > 1 indicated antagonism, CI = 1 indicated summation, CI < 1 indicated synergism.

The equation of median effect method was shown as following:

$$\mathrm{CI} = \frac{(D_x)_1}{(D)_1} + \frac{(D_x)_2}{(D)_2} + a\frac{(D_x)_1(D_x)_2}{(D)_1(D)_2}$$

$$\frac{F_a}{F_u} = \left[\frac{D}{D_m}\right]^m$$

F_a = fraction of system affected by drugs,
F_u = fraction of system unaffected by drugs, $F_u + F_a = 1$
D_m = dose of drug required to produce a median effects (IC_{50}).
m = slope of the median-effect plot, in which $y = \lg(F_a/F_u)$, $x = \lg(D)$
$(D_x)_{1,2}$ = the dose of drugs treated in combination pattern
$(D)_{1,2}$ = the dose of drugs used alone

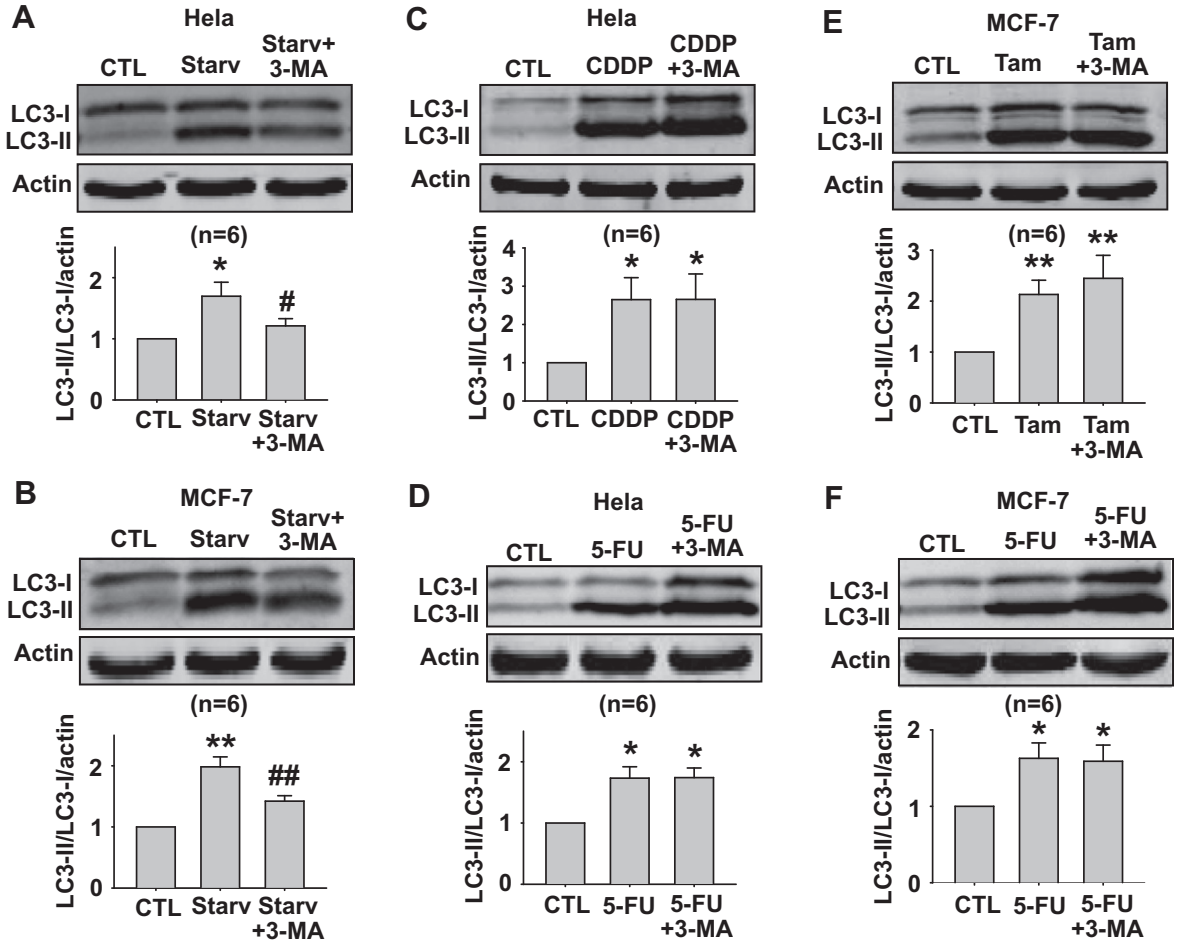

Fig. 3. 3-MA inhibited starvation-induced rather than chemotherapeutic drug-induced autophagy. (A and B) 3-MA(5 mM) inhibited starvation-induced autophagy in Hela and MCF-7 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. starv. Starv, starvation (serum-free DMEM with 5.5 mM glucose, 24 h). (C and D) 3-MA(5 mM) did not inhibit CDDP(20 μM) and 5-FU(75 μM)-induced autophagy in Hela cells. $^{*}P < 0.05$ vs. control. (E and F) 3-MA(5 mM) did not inhibit tamoxifen (10 μM) and 5-FU(75 μM)-induced autophagy in MCF-7 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. Cells were treated for 24 h.

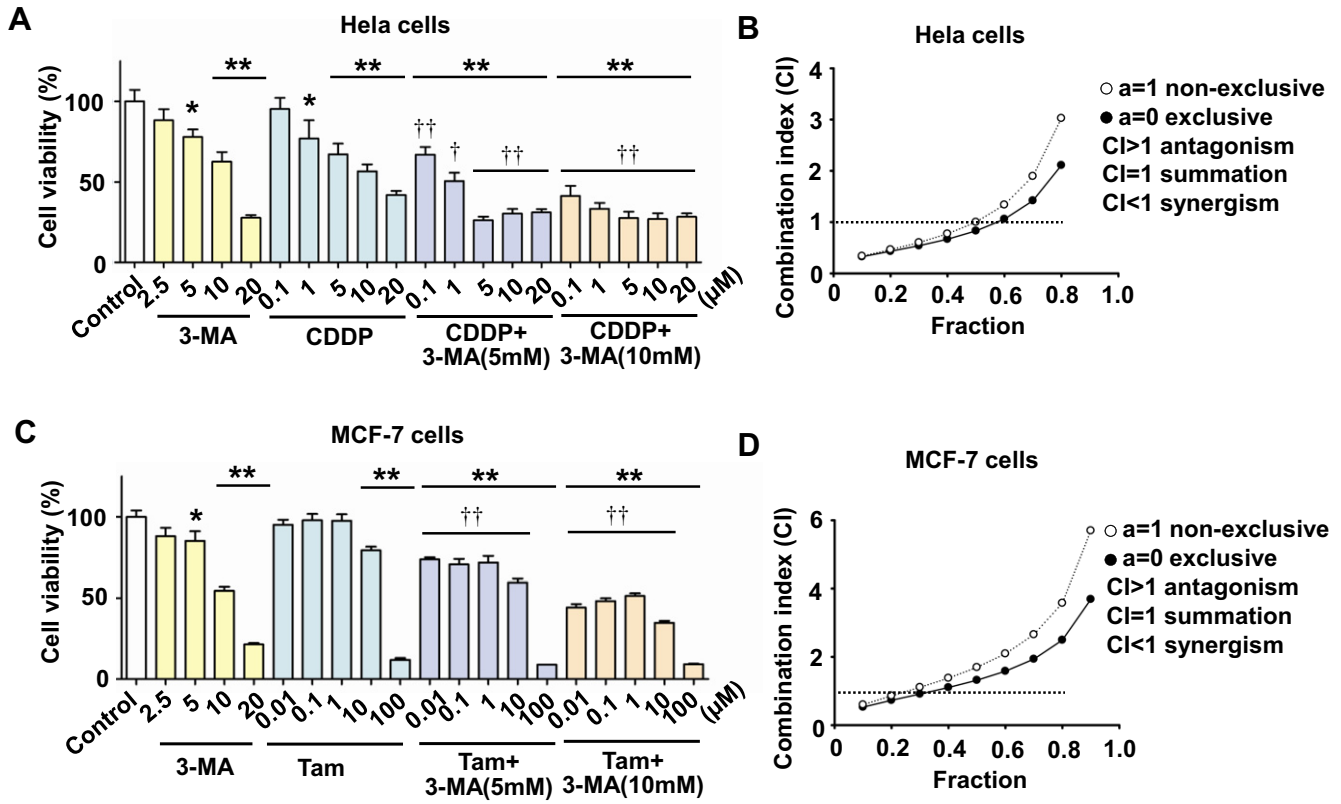

Fig. 4. The combined effects of 3-MA and CDPP/tamoxifen on Hela and MCF-7 cell viability. (A) Effects of 3-MA,CDDP and combination of 3-MA with CDDP on Hela cell viability after 24 h treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. CDDP, CDDP, cispcis-diamminedichloroplatinum(II). $n = 6$. (B) The combination index showed that 3-MA synergized the inhibitory effects of CDDP on Hela cells in the range of 0 to 0.6 fraction but antagonized when the fraction was more than 0.6. (C) Effects of 3-MA, tamoxifen and combination of 3-MA with tamoxifen on MCF-7 cell viability after 24 h treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. Tam, tamoxifen. $n = 6$. (D) The combination index showed that 3-MA synergized the inhibitory effects of tamoxifen on MCF-7 cells in the range of 0 to 0.3 fraction but antagonized when the fraction was more than 0.3 fraction.

2.8. Data analysis

Data are presented as mean ± SEM. Significance was determined by using Student *t* test or one-way ANOVA, followed by Tukey post test. $P < 0.05$ was considered significant.

3. Results and discussion

As shown in Fig. 1A and C, MA dose-dependently reduced Hela and MCF-7 cell viability after 24 h treatment. Then, cell autophagy was evaluated by measuring LC3-II which was recruited to autophagosomal membranes, reflecting autophagic activity [18]. In the normal culture conditions, 3-MA at concentration of 5 mM which is commonly used for inhibiting autophagy showed no effect on the basal autophagy responses in Hela and MCF-7 cells (Fig. 1B and D). Since 3-MA reduced cell viability, we further examined the effects of 3-MA on cell death and apoptosis. Live and dead assay showed that 3-MA at 5 and 10 mM concentrations significantly induced Hela cell death (Fig. 2A). TUNEL staining showed that 3-MA at 5 and 10 mM concentrations also induced Hela cell apoptosis (Fig. 2B). These data suggested that 3-MA induced cell death and apoptosis and this induction was not related to autophagy. Consistent with the present data, a recent work showed that 3-MA decreased cell viability in a time- and dose-dependent manner and 3-MA-induced cell death was not affected by knockdown of beclin-1, namely, 3-MA induced cell death is independent of its ability to inhibit autophagy [19].

3-MA is widely used as an autophagy inhibitor. Indeed, 3-MA inhibited starvation-induced autophagy in Hela and MCF-7 cells (Fig. 3A and B), consistent with the previous study [20,21]. However, 3-MA did not inhibit the increased autophagy responses induced by chemotherapeutic drugs CDDP, tamoxifen and 5-FU in Hela and MCF-7 cells (Fig. 3C–F).

Then, we studied the effects of combination of 3-MA with CDDP/tamoxifen on cell viability by using MTT method. As shown in Fig. 4A, CDDP dose-dependently reduced Hela cell viability and combination use of 3-MA further enhanced CDDP-induced reduction of cell viability. Similarly, tamoxifen dose-dependently reduced MCF-7 cell viability and combination use of 3-MA further enhanced tamoxifen-induced reduction of MCF-7 cell viability (Fig. 4C). In order to identify the summation, synergism and antagonism interaction between 3-MA and anti-cancer drugs, we used MTT method to measure the cell viability and used the median effect methods to evaluate the interaction between 3-MA and CDDP/tamoxifen. As shown in Fig. 4B, 3-MA synergized the inhibitory effects of CDDP on Hela cells in the range of 0 to 0.6 fraction (inhibition ratio) but antagonized when the fraction was more than 0.6. As shown in Fig. 4D, 3-MA synergized the inhibitory effects of tamoxifen on MCF-7 cells in the range of 0 to 0.3 fraction but antagonized when the fraction was more than 0.3 fraction. These results suggested that the synergism or antagonism between 3-MA and chemotherapeutic drugs was dependent on the inhibition ratio of tumor cells. Together with the results that 3-MA did not inhibit the increased autophagy responses induced by chemotherapeutic drugs (Fig. 3C–F), it indicated that the interaction between 3-MA and chemotherapeutic drugs was not related to autophagy.

Several studies have found that anti-cancer drugs induced autophagy response which was inhibited by 3-MA, for example, 3-MA inhibited paclitaxel-induced autophagy in A549 cells [22], inhibited 5-FU-induced autophagy in human colon cancer cell lines [11]. Based on the correlativity between autophagy inhibition and cell viability, it has been established that inhibition of autophagy augments chemotherapy efficacy [6]. However, in the present work, we did not find that 3-MA inhibited CDDP, tamoxifen and 5-FU induced autophagy in Hela and MCF-7 cells. Ito et al. found that 3-MA suppressed cell migration and invasion of HT1080 fibrosarcoma cells independently of autophagy inhibition [23], similarly to the present data. We conclude that 3-MA itself induces cell death and apoptosis without relationship with autophagy; 3-MA does not inhibit the increased autophagy induced by anti-cancer drugs; the interaction between 3-MA and chemotherapeutic drugs is not related with autophagy.

Acknowledgments

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