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Requirement of SIRT1-mediated FoxO1 activation for Multidrug Resistance-Associated Protein 2 expression in Tamoxifen resistant breast cancer cells

조선대학교 대학원

약학과

조 경 빈

타목시펜 저항성 유방암 세포에서 MRP2를 발현 시키는 FoxO1 활성에 대한 SIRT1의 역할

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(국문 초록)

타목시펜 저항성 유방암 세포에서 MRP2 를 발현 시키는 FoxO1 활성에 대한 SIRT1 의 역할

조 경 빈

지도 교수 : 강 건 욱

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항암제 치료 시 발생하는 화학요법 반응성 유방암 세포에서 화합요법 저항성 암세포로의 전환에는 ATP 의존적 수송체인 ABC transporter 의 발현 증가가 흔히 수반된다. 이 논문에서 본인은 FoxO1 전사인자가 MDR1 뿐만 아니라 MRP2 의 발현을 조절하고, SIRT1 이 이러한 FoxO1 의 조절을 통해 MRP2 과다발현에 관여하고 있음을 규명하였다. 아울러, SIRT1 의 inhibitor 인 Amurensin G (CPP343)이 화학요법 저항성 유방암 세포에서 MRP2 와 MDR1 의 발현을 억제시킴을 보였다. MRP2 유전자의 proximal promoter 부위에서 FoxO 결합부위로 추정되는 부위를 발견하였고, 대조군 유방암 세포인 MCF-7 세포에 비해서 MRP2 와

FoxO1 의 발현 양이 증가되어 있는 TAMR-MCF-7 세포에서 MRP2 promoter 의 전사활성이 크다는 것을 확인하였다. 이렇게 증가되어 있는 MRP2 의 발현과 전사활성화는 FoxO1 siRNA 에 의해 억제되었다. 또한 TAMR-MCF-7 세포에서는 SIRT1 의 발현 및 효소의 활성이 MCF-7 세포에 비해 훨씬 높으며 SIRT1 inhibitor 인 nicotinamide 처치 시 TAMR-MCF-7 세포에서 FoxO1 와 MRP2 의 발현이 억제되었다. FoxO1 과 SIRT1 을 과다 발현시켰을 때 MDR1 과 MRP2 모두의 발현과 전사활성이 늘었고 이것은 다시 FoxO1 siRNA 에 의해 억제되었다. 강력한 SIRT1 억제효과를 보이는 천연 화합물인 CPP343 에 의해 TAMR-MCF-7 세포와 MCF-7/ADR 세포에서 각각 MPR2, MDR1 의 발현이 억제되었다. 또한, MCF-7/ADR 세포에 CPP343 을 처치 시 MDR1 transporter 의 기질인 doxorubicin 이 세포 내에 유지되는 양이 증가하였고, doxorubicin 에 대한 반응성이 회복되었다. 이러한 결과를 통해 SIRT1 이 FoxO1 을 매개한 MRP2 의 발현에 있어 중요한 역할을 하며 SIRT1 의 조절을 통해 화학요법 저항성의 치료에 도움을 줄 수 있음을 시사하고 있다.

ABSTRACT

Requirement of SIRT1 as critical regulator for Multidrug Resistance−Associated Protein 2 expression

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The transition from chemotherapy-responsive breast cancer cells to chemotherapy-resistant cancer cells is mainly accompanied by the increased expression of ATP-binding cassette transporters. Multi-drug resistance protein 2 (MRP2, ABCC2) plays an important role in the efflux of xenobiotics or conjugated metabolites. In our previous study, it has been found that FoxO1 functions as a key regulator of *MDR1* gene transcription (1). Because the mechanism how MRP2 protein is up-regulated during chemotherapy-resistance acquisition has been rarely studied, in the present study, we aimed to

clarify whether FoxO1 control the expression of *MRP2* gene in tamoxifen-resistant breast cancer cells. Moreover, based on the fact that nuclear localization of FoxO1 is regulated by SIRT1 deacetylase, we were further interested in whether a potent SIRT1 inhibitor, Amurensin G (CPP343) identified from screening of natural compound library, inhibits the expressions of MRP2 and MDR1 in chemotherapy-resistant breast cancer cells. Overexpression of FoxO1 and/or SIRT1 enhanced MRP2 protein level and the gene transcriptional activity. In addition, SIRT1 inhibition reduced both the nuclear FoxO1 levels and the MRP2 expression in tamoxifen-resistant MCF-7 (TAMR-MCF-7) cells. MDR1 was also proved to be regulated by SIRT1-dependent FoxO1 activation as well. CPP343, isolated from *Vitis amurensis* suppressed both the basal expression of MDR1 in adriamycin-resistant MCF-7 (MCF-7/ADR) cells and that of MRP2 in TAMR-MCF-7 cells. Moreover, pretreatment of MCF-7/ADR cells with CPP343 for 24 h significantly increased cellular uptake of doxorubicin and restored doxorubicin responsiveness in MCF-7/ADR cells. These results suggest that FoxO1 activation via SIRT1-dependent deacetylation is closely related with up-regulations of MRP2 and MDR1. Furthermore, CPP343, a natural SIRT1 inhibitor could be developed as a chemotherapy

adjuvant to increase the susceptibility of anti-cancer agents in chemoresistant cancer.

1. Introduction

Although chemotherapy is the widely used device for the treatment of various cancers, its use is frequently limited by the multidrug resistance (MDR) acquisition in tumor cells. MDR describes the phenomenon of resistance not only to the current drug but also to structurally different chemopreventive agents (2). One of the most important mechanisms for cancer chemotherapy resistance is the active efflux of anti cancer drugs through the increased expression of the drug transporters (3, 4). There are well-known three MDR genes identified in humans; multidrug resistance-associated proteins (MRPs, ABCC subfamily), MDR1 (p-glycoprotein, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) (5, 6). All are members of the ATP-binding cassette (ABC) transporter family. ABC transporters are transmembrane proteins that function in the pumping out of a number of substrates across cellular membranes by ATP binding. Though there may be some differences among them, these transporters commonly have been known to efflux a variety of anti-tumor agents (7). However, the expression mechanisms of ABC transporters have not been fully understood despite of obvious expression of these proteins in most

tumor tissues. Moreover, attempts to modulate the activity of these proteins have met with limited success (2).

Incidence of breast cancer is rising in women throughout the world, as well as western women nowadays. Administration of anti-estrogen is most common therapeutic method. A non-steroidal anti-estrogen, tamoxifen (TAM), an orally active selective estrogen receptor modulator (SERM), has been approved for the chemoprevention of breast cancer and is the most widely used anti-estrogen in estrogen receptor-positive breast cancer patients (8). Although most patients are initially responsive, the acquisition of resistance to TAM is the main problem of anti-estrogen therapy (9). We have previously shown that the overexpression of MRP2 and MRP1 was found in TAM-resistant MCF-7 cells (TAMR-MCF-7 cells) and have suggested that the up-regulation of ABC transporters plays a role in the additional acquisition of chemotherapy resistance (10).

Forkhead box-containing protein, O subfamily (FoxO) transcription factors hold a conserved DNA binding domain termed the Fokrhead box (Fox). Four proteins FoxO1, FoxO3, FoxO4 and FoxO6 are found to be the member of O subfamily FoxO in mammals. Transcriptional activity of FoxO factors is regulated by a shuttling

system running between the nucleus and the cytoplasm and this can be regulated by phosphorylation-dependent ubiquitination and acetylation (11, 12, 13). A variety of cellular fates such as differentiation, metabolism and proliferation are controlled by FoxO (14), and FoxO factors are frequently mis-regulated in some cancers (13, 15). We recently found that FoxO1 is consistently up-regulated in adriamycin-resistant breast cancer cells, which plays a critical role in the expression of *MDR1* gene (1).

Silent information regulator two ortholog 1 (SIRT1) is the human ortholog of the yeast sir2 protein, which is the name of a family of closely related enzymes, the sirtuins (16, 17). Sirtuins play a key role in cellular response to stresses such as heat or starvation and are responsible for the lifespan extending processes of calorie restriction (16, 18). Sirtuins act by removing acetyl groups from proteins in the presence of NAD+; they are thus classified as NAD+-dependent deacetylases (18). Several transcription factors (e.g., p53, myoD, NFκB) have been reported as substrates of SIRT1 (18, 19). FoxO transcription factors are also deacetylated by SIRT1 and consequently accumulated in nucleus (20).

Based on a hypothesis that SIRT1-dependent FoxO1 activity is

important for the expression regulation of ABC transporters, we tried to clarify potential role of SIRT1 activation in the up-regulation of MRP2 in TAM-resistant breast cancer cells. Moreover, we also assessed the effect of Amurensin G (CPP343), a potent SIRT1 inhibitor isolated from *Vitis amurensis*, on the MDR1 and MRP2 expressions in chemotherapy-resistant breast cancer cell types (MCF-7/ADR and TAMR-MCF-7 cells).

2. Materials and Methods

2-1. Materials

The anti-MDR1 antibody was supplied by Calbiochem (Darmastadt, Germany). The FoxO1 and FoxO3a specific antibodies, phosphorylated Akt and Akt antibodies, SIRT1 antibody, the horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs were purchased from Cell Signaling Technology (Beverly, MA). The antibody against MRP2 was supplied by Santa Cruz Biotechnology (Santa Cruz, CA) and the alkaline phosphatase-conjugated donkey anti-mouse IgG and the horseradish peroxidase-conjugated rabbit anti-goat IgG were provided by Jackson Immunoresearch Laboratories (West Grove, PA). Most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). The siRNA targeting human FoxO1 was acquired from Ambion (Austin, TX). Human recombinant SIRT1, Fluor de Lys SIRT1 deacetyalse substrate, Fluor de Lys Developer Ⅱ, NAD+ and the buffer used for assays were purchased from Biomol (Plymouth Meeting, PA) and CPP342, CPP343 were kindly provided by Dr. WK Oh (Chosun university, Gwang-ju, Korea).

2-2. Cell culture

The MCF-7 cells and the adriamycin-resistant MCF-7 (MCF-7/ADR) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5 % CO_2 humidified atmosphere. TAM-resistant MCF-7 cells (TAMR-MCF-7) were established using the methodology reported elsewhere (Knowlden et al. 2003). Briefly, MCF-7 cells were washed with PBS, and the culture medium was changed to phenol-red-free DMEM containing 10% charcoal-stripped, steroid-depleted fetal bovine serum (Hyclone, Logan, UT) and 4-hydroxytamoxifen (0.1 μM). The cells were continuously exposed to this treatment regimen for 2 weeks and the concentration of 4-hydroxytamoxifen was gradually increased to 3 μM over a 9-month period. Initially, the cell growth rates were reduced. However, after exposure to the medium for 9 months, the rate of cell growth gradually increased, showing the establishment of a tamoxifen-resistant cell line (10).

2-3. Plasmids

The p2635-MRP2-Luc or p491-MRP2-Luc reporter plasmid containing human MRP2 promoter region (-2635 and -491 bp, respectively) was kindly provided by Dr. Uchiumi T (Kyushu University, Fukuoka, Japan) (21). The p195-MDR1 reporter plasmid was generated by ligating PCR-amplified MDR1 promoter regions with pGL3-enhancer vector (Promega, Madison, WI). The pcDNA3-FoxO1 and pcDNA3-FoxO3 overexpression plasmids were supplied from Addgene Inco. (Cambridge, MA). The SIRT1 constitutive active plasmid was kindly donated by Dr. KY Lee (Chonnam National University, Gwangju, Korea).

2-4. Preparation of nuclear extracts

Nuclear extracts were prepared essentially as described by Schreiber et al (22). Briefly, cells in dishes were washed with ice-cold PBS, scraped, transferred to microtubes, and allowed to swell after adding 100 μl of lysis buffer containing 10 mM HEPES (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonylfluoride (PMSF). Cell membranes were disrupted by vortexing, and the lysates were incubated for 10 min on ice and centrifuged at 7,200g for 5 min. Pellets containing crude

nuclei were resuspended in 60 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride, and then incubated for 30 min on ice. The samples were then centrifuged at 15,800g for 10 min to obtain supernatants containing nuclear extracts, which were stored at -80°C until required.

2-5. Immunoblot analysis

After washing with sterile PBS, the MCF-7, TAMR-MCF-7 or MCF-7/ADR cells were lysed in EBC lysis buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM -glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. The cell lysates were centrifuged at 10,000*g* for 10 min to remove the debris, and the proteins were fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with the specific antibodies. Horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibodies were used as the secondary antibodies. The

nitrocellulose papers were developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/4-nitroblue tetrazolium (NBT) or an ECL chemiluminescence system. For chemiluminescence detection, the LAS3000-mini (Fujifilm, Tokyo, Japan) was used.

2-6. SIRT1 Deacetylase Assay

The Fluor de Lys fluorescence assay was used to assess activity of several compounds and SIRT1 enzyme activation in cell extracts. The Fluor de Lys fluorescence assay was performed as indicated in the BioMol product sheets (16). Briefly, assays were performed using Fluor de Lys-SIRT1, NAD+, SIRT1 or cell extracts in SIRT1 assay buffer (50 mM Tris-Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mg/ml bovine serum albumin, as indicated in the BioMol product sheets.). The mixture of 10 mM NAD+, 5 mM substrate, SIRT assay buffer was preincubated. Total cell lysates were added instead of SIRT1 enzyme. Prior to quenching the reaction, 2 mM nicotinamide was added to 1×Developer Ⅱ in the histone deacetylase assay buffer (50 mM Tris, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, as indicated in the BioMol product sheets). After adding the Developer solution for quenching, the samples were kept at 37℃ for 45 min prior

to fluorescence reading. The fluorescence was measured using the excitation and emission wavelengths of 360 and 450 nm, respectively.

2-7. MTT cell viability assay

To determine the cell viabilities, the cells were plated at 10^4 cells/well in 96-well plates. For the cytotoxicity determination by CPP343, TAMR-MCF-7, and MCF-7/ADR cells were incubated in the FBS-free medium with or without CPP343 (0.1–3 μg/ml) for 24 h. The viable adherent cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; 2 mg/ml) for 4 h. The media were then removed and the formazan crystals produced were dissolved by adding 200 μl dimethylsulfoxide/well. The absorbance was assayed at 540 nm. The cell viability was expressed as the relative ratios to the untreated control cells.

2-8. Crystal violet assay

Cell viability was determined by crystal violet staining when the cells were treated with doxorubicin (23). Cells were stained with 0.4% crystal violet in methanol for 30 min at room temperature and then washed with tap water. Stained cells were extracted with 50% methanol

and dye extracts were measured at 550 nm wavelength using a microtiter plate reader (Berthold Technologies, Bad Wildbad, Germany).

2-9. Cellular uptake of doxorubicin

The transport of doxorubicin was quantified in MCF-7 and MCF-7/ADR cells. Cells ($3x10^6$ cells) were incubated with 30 μM doxorubicin for 60 min, washed with PBS three times, and lysed in EBC lysis buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. After centrifugation of the samples at 10,000*g* for 10 min, change in fluorescent absorbance of doxorubicin in the supernatant was determined at the excitation and emission wavelengths of 470 nm and 590 nm, respectively. The uptake intensity was expressed as relative ratio to the fluorescence value of doxorubicin-treated group.

2-10. TdT mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay were performed using an *in situ* cell death

detection kit (Roche Diagnostics GmbH, Germany). After 18 h incubation with either doxorubicin (30 μM) or CPP343 (0.3-1 μg/ml), MCF-7 or MCF-7/ADR cells were washed with phosphate buffered saline (PBS). Cells on slides were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature, and then permeabilized with 0.1% Triton® X-100 in 0.1% sodium citrate for 2 min on ice. They were then washed with PBS, incubated for 60 min at 37°C after adding 50 μl of TdT enzyme solution, incubated for 30 min at 37°C after adding 50 μl of anti-fluorescent antibody (Fab fragment from sheep conjugated with alkaline phosphatase), and further incubated for 10 min in the presence of BCIP/NBT solution. Slides were then rinsed with phosphate-buffered saline, mounted under cover-slips, and analyzed under an optical microscope.

2-11. Reporter gene assay

The promoter activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, the cells (3×10^5 cells/well) were replated in 12-well plates overnight and transiently transfected with the p2635-MRP2-Luc or p491-MRP2-Luc, p-MDR1 Luc reporter plasmids/phRL-SV plasmid (*hRenilla* luciferase

expression for normalization) (Promega, Madison, WI) using Hilymax® reagent (Dojindo Molecular Technologies, Gaithersburg, MD). The cells were then incubated in the culture medium without serum for 18 h, and the firefly and hRenilla luciferase activities in the cell lysates were measured using a luminometer (LB941, Berthold Technologies, Bad Wildbad, Germany). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity versus hRenilla luciferase.

2-12. Statistical analysis

Paired Student's t-test was used to examine the significant inter-group differences. Statistical significance was set at either $p<0.05$ or $p<0.01$.

3. Results

3-1. Novel role of FoxO1 in the *MRP2* gene transcription

There are many transcription factors binding sites in human MRP2 promoter region (21). AP-1, HNF-3β, SP-1, and C/EBPβ are the examples of transcription factors binding to the MRP2 promoter (Figure 1A). We revealed that the exaggerated PXR activity was required for the oevrexpression of MRP2 in TAMR-MCF-7 cells, but C/EBPβ binding was not responsible for the transactivation of *MRP2* gene (10). We also demonstrated that FoxO1 binding site in MDR1 promoter is essential for the basal expression of *MDR1* gene in adriamycin-resistant breast cancer cells (MCF-7/ADR cells) (1). As shown in Figure 1A, we also found several putative FoxO binding sites that closely match consensus sequence in human MRP2 promoter region (13, 24).

To determine whether FoxO binding to the putative binding sites is related with *MRP2* gene transcription, we measured the effects of FoxO1 and FoxO3 overexpression on MRP2 expressions, using MCF-7 cell lines. The expression levels of MRP2 were increased in the MCF-7 cells transfected with FoxO1 overexpressing vector

(pcDNA3-FoxO1) (Figure 1B). In contrast, MRP2 protein levels were rarely elevated by FoxO3 overexpressing plasmid and the induction intensity was much weaker than that by FoxO1.

We next tested whether FoxO1 introduction stimulates the MRP2 promoter activity. In MCF-7 cells, the human p2635-MRP2-Luc reporter activity was significantly increased by FoxO1 overexpressing plasmid in a concentration dependent manner (Figure 1C). These results demonstrate that FoxO1 functions as a key transcription factor in the expression of MRP2 as well as MDR1.

We have suggested that the sustained MRP2 up-regulation in TAM-resistant breast cancer cells is presumably associated with the additional chemotherapy resistance acquisition (10). As shown in Figure 2A, we confirmed that the basal MRP2 levels were higher in the TAMR-MCF-7 cells than control MCF-7 cells (Figure 2A). When we measured the FoxO1 protein levels using the same samples, the FoxO1 amounts in the total cell lysates obtained from TAMR-MCF-7 cells were distinctly higher than those in MCF-7 cells. However, the basal FoxO3 levels were not changed in TAMR-MCF-7 cells (Figure 2A). Because nuclear localized FoxO only acts as functional transcription factor, nuclear FoxO1 levels were also compared between the two cell

types. In comparison to control MCF-7 cells, nuclear levels of FoxO1, but not those of FoxO3, were sharply increased in TAMR-MCF-7 cells (Figure 2A).

Reporter gene analyses were performed using two types of hMRP2-Luc Plasmids; p2635-MRP2-Luc and p491-MRP2-Luc, which contained a -2.6kb and -491bp human MRP2 promoter region, respectively. Despite of deletion, p491-MRP2-Luc still retains remaining putative FoxO binding sites with rarely destruction, while C/EBPβ binding regions were truncated. Though relative intensity gets lower at p491-MRP2, the tendency of luciferase activity was similar at both of MRP2 promoters (Figure 2B). This suggests that FoxO1, not C/EBPβ may have an important role in regulation of MRP2.

To confirm the role of FoxO1, western blot analysis was performed in the TAMR-MCF-7 cells applied with small inhibitory RNA (siRNA) specifically silencing FoxO1. When FoxO1 expression was inhibited by FoxO1 siRNA, the elevated MRP2 levels were reversed (Figure 2C). Reporter gene assay with p491-MRP2-Luc conserving FoxO1 binding sites was next performed in MCF-7 and TAMR-MCF-7 cells co-transfected with FoxO1 siRNA. The MRP2 reporter activity was dramatically diminished by FoxO1 inactivation

(Figure 2C). These data suggest that FoxO1 is a critical regulator for human *MRP2* gene expression and transcriptional activity.

3-2. Involvement of SIRT1 in the MRP2 and MDR1 expressions

FoxO proteins can be controlled by two different mechanisms; phosphorylation and acetylation. Multiple kinase pathways including PI3K/Akt, MEK/Erk, JNK have shown to regulate FoxO via phosphorylation (12, 25, 26). In addition, it has been also reported that SIRT1 causes nuclear translocation of FoxO1 by its deacetylation and subsequently increases the transcriptional activity of FoxO1 (27).

Activated Akt is known to phosphorylate FoxO1 proteins, leading to inhibition of Foxo1 transcriptional activity by exporting it to cytoplasm from the nucleus (12, 28). The levels of phosphorylated Akt were higher in TAMR-MCF-7 cells than MCF-7 cells, which is consistent with our previous result (Figure 3A). Because Akt activation decreases the nuclear expression and transactivator function of FoxO1, this result seems to be conflict with the result that nuclear FoxO1 was relatively higher in TAMR-MCF-7 cells than control MCF-7 cells, shown in Figure 2. Thus, consistent Akt activation in TAMR-MCF-7 cells seems not to be related with the enhanced nuclear FoxO1, as

confirmed by Akt inhibition (Figure 3C). Instead, SIRT1 expression was higher in TAMR-MCF-7 cells than in MCF-7 cells (Figure 3B). To clarify it, we further determined SIRT1 enzyme activity using Fluor de Lys fluorescent substrate in the total cell lysates. TAMR-MCF-7 cells showed higher SIRT1 activity than MCF-7 cells (Figure 3B). On the other hand, MCF-7/ADR cells where MDR1 level is highly expressed, showed lower SIRT1 activity than control MCF-7 cells (Figure 3B). Instead, phosphorylated Akt levels were down-regulated in MCF-7/ADR cells (Figure 3A). We assume that overexpression of FoxO1 in MCF-7/ADR is due to the poor regulatory capability of Akt. These results strongly support a notion that SIRT1 acts as a key regulator of FoxO1 activity in TMAR-MCF-7 cells.

We then examined whether SIRT1 activity is essential for the FoxO1-dependent MRP2 expression. Nicotinamide, a representative SIRT1 inhibitor, proved to have an inhibitory effect on FoxO1 according to the results of both nuclear levels and total cell lysates protein levels (Figure 3C). Nicotinamide also distinctly reduced the level of MRP2 protein in TAMR-MCF-7 cells (Figure 3C). PI3-kinase inhibition (LY294002-treated group) also reduced the basal MRP2 levels in TAMR-MCF-7 cells, but the nuclear FoxO1 levels were not

affected (Figure 3C). In our previous study, we showed that PI3-kinase/Akt activation up-regulates MRP2 protein through PXR dependent manner (10). Hence, FoxO1-dependent MRP2 expression would mainly result from SIRT1 activation in TAMR-MCF-7 cells. SIRT1 inhibition also decreased *MRP2* gene transcription (Figure 4A). Based on these results, the effects of SIRT1 overexpression on MRP2 were investigated. The transcription of *MRP2* genes was increased in accordance with SIRT1 addition (Figure 4B). Because FoxO1 overexpression also stimulated *MDR1* gene transcription in our previous results and we aimed to clarify a link between FoxO1-mediated MDR1 and SIRT1, the effect of SIRT1 on MDR1 was tested. The p195-MDR1-Luc reporter activity was significantly elevated by SIRT1 constitutive active plasmid in MCF-7 cells (Figure 4B). Besides, SIRT1 also enhanced transcriptional activity of forkhead-response element (FHRE) (Figure 4C). These results definitely imply that SIRT1 is closely connected with the expression of FoxO1-mediated MDR proteins.

3-3. Synergistical effects of SIRT1 on FoxO1-dependent MRP2 and MDR1 overexpression

To determine certainly whether SIRT1 overexpression potentiates FoxO1-mediated *MRP2* and *MDR1* gene transcription, MCF-7 cells were co-transfected with constitutive active SIRT1 plasmid and/or FoxO1 overexpression plasmid. SIRT1 overexpression in the presence of FoxO1 synergistically enhanced both of MRP2 and MDR1 promoter activities, whereas each one also showed MRP2 and MDR1 increasing effect (Figure 5A, 1C and 4B). Furthermore, SIRT1-induced MRP2 and MDR1 transactivation was reversed by FoxO1 siRNA (Figure 5B). These data support that SIRT1 is a critical regulator for MRP2 expression as well as MDR1 via FoxO1 dependent mechanism and FoxO1 and SIRT1 aid each other to induce multidrug resistance expression.

3-4. Effects of natural SIRT1 inhibitors, CPP342 & 343 on MRP2 and MDR1 expression

SIRT1 is believed as one of attractive anti-cancer targets, since SIRT1 controls cell cycle and apoptosis during tumorigenesis. Although several SIRT inhibiting chemicals have been identified, potential toxicity is a main obstacle to develop new anti-cancer agent. Hence, we tried to identify potent SIRT1 inhibitors from natural

product compounds library. Among the screening data of 400 species, CPP342 (Ampelopsin) and CPP343 (Amurensin G) showed potent inhibitory effects against SIRT1 enzyme activity and especially CPP343 showed more potent activity than CPP342 (Oh et al. unpublished data) (Figure 6A and data is not shown). In the first place, we performed preliminary experiment about the efficiency of CPP343 in MCF-7 cell. After enhancing FHRE reporter activity using constitutive active SIRT1 plasmid, CPP343 was treated in MCF-7 cells. CPP343 reduced SIRT1-induced FHRE transcriptional activities in a concentration dependent manner (Figure 6B). It could be evidence supporting potentiality of natural substances as SIRT1 inhibitor for in vivo methodology. In order to determine whether or not the SIRT1 inhibitors generally suppress ABC transporters, we studied these natural product compounds. Western blot analysis was performed to elucidate the effects of these compounds on MDR1 and MRP2 expressions. Either CPP342 or CPP343 significantly inhibited the expression of MDR1 and MRP2 proteins in MCF-7/ADR cells and TAMR-MCF-7 cells, respectively (Figure 6C, 6D). Moreover, nuclear FoxO1 levels were attenuated by CPP343 treatment, while FoxO3 was not altered by CPP343 (Figure 6D). To confirm its applicative effects,

we performed preliminary experiments on the cytotoxicity of CPP343. CPP343 seems not to have severe toxicity in both of the TAMR-MCF-7 and MCF-7/ADR cells (Figure 7A). Next, we tested whether the cellular uptake of doxorubicin was enhanced by CPP343. Doxorubicin was accumulated in MCF-7/ADR cells to a lesser extent than in MCF-7 cells. Doxorubicin uptake in MCF-7/ADR cells was enhanced as increasing concentration of CPP343 (Figure 7B). We further determined whether doxorubicin responsiveness is recovered by CPP343 treatment. After 24 h preincubation of MCF-7/ADR cells with vehicle or CPP343 (0.1, 0.3, 1 μg/ml), doxorubicin (30 μM)-mediated cell viability change was assessed. CPP343 treatment significantly enhanced the cytotoxicity of doxorubicin, as evidenced by crystal violet assay (Figure 7C). We finally assessed doxorubicin-induced apoptosis in MCF-7/ADR cells after CPP343 treatment. Representative terminal dUTP nick-end labeling (TUNEL) assay photographs showed that exposure of control MCF-7 cells to doxorubicin (30 μM) for 24 h caused severe apoptosis, but not in MCF-7/ADR cells. However, TUNEL-positive cells were found in MCF-7/ADR cells pretreated with CPP343 (0.3-1 μg/ml) for 24 h suggesting the down-regulatory effect of CPP343 on MDR1 activity and the recovery of sensitivity to

doxorubicin. (Figure 7D).

4. Discussion

Multi-drug resistance is a serious obstacle in the treatment of breast cancer (29). Several ATP-binding cassette (ABC)-superfamily multidrug efflux pumps are known to be involved in this phenomena. p-glycoprotein (MDR1, ABCB1), MRP2 (cMOAT, ABCC2) are the overt protein to drive this severe resistance (30, 31). Although these efflux systems can be of help to removing harmful chemicals and protecting tissues from toxic materials, they have heavier problem to manage the patients who are taking drugs deciding their fate at the crossroads of life and death. What is worse, these proteins have intersecting effects on other unrelated anti-cancer drugs, bring about the low success of treatment (32). Among the ABC transporters, MRP2 protein is reported to be overexpressed in malignant neoplastic tissues (33). In addition to transporting of endogenous conjugates, MRP2 transports cancer chemotherapeutics. Anticancer drugs such as vinblastine, vicristine and methotrexate are substrates of MPR2; therefore this protein appears to contribute to drug resistance in mammalian cells (33, 34, 35, 36). However, the molecular mechanisms by which MRP2 is increased are largely unknown. In our previous

study, up-regulation of C/EBPβ was discovered on the process of establishing the chemotherapy-resistant cancer cell lines. But it was concluded that C/EBPβ may not be responsible for *MRP2* gene activation (10).

Because there are many number of putative FoxO transcription factor binding sites on MRP2 promoter region, we brought FoxO into focus. In the present study we showed that there was close correlation between MRP2 and FoxO1 transcription factor. When we compared the MRP2 levels in FoxO1 overexpressed human breast cancer cell lines, MRP2 protein levels were elevated. Remarkably enhanced FoxO1 levels were observed at TAMR-MCF-7 cells, which have canonically overexpressed MRP2 proteins. Even if we also deliberated on FoxO3, its intensity was weaker than FoxO1.

Keiji et al. (2004) showed that compared with p1659 MRP2, the luciferase activity decreased when p491 MRP2 was assayed in HepG2 cells, leading to the conclusion that a putative positive regulator can be localized in the -1659/-491 bp region (37). On the contrary to this results, Tanaka et al. previously took the results that the luciferase activity decreased in HepG2 cells when p1659 MRP2 was assayed, compared with p491 MRP2, concluded by saying that there was a

putative silencer regulatory element in the -1659/-491 region (21). The conclusions of these two studies are exactly contrary to each other. To solve this conflict, more detailed investigation is needed. Among such complex elements, FoxO1 proved to be the positive regulator of MRP2. Although a part of FoxO1 binding region is truncated in p491 MRP2, its loss is not greater than C/EBPβ and p491 MRP2 probably still reserve another several binding sites for FoxO1. Therefore p491 MRP2 is also shown to play a role in increasing the MRP2 luciferase activity in TAMR-MCF-7 cells compared with MCF-7 cells.

Further study was performed by introducing FoxO1 specific siRNA. When we experimented on western blot assay, FoxO1 interfering by siRNA restrained MRP2 protein expression in TAMR-MCF-7 cells. Reporter gene assay results also showed similar tendency. After co-transfected the MCF-7 cells and TAMR-MCF-7 cells with FoxO1 siRNA, we measured MRP2 reporter activity. The activity of MRP2 levels was reduced drastically by FoxO1 siRNA in TAMR-MCF-7 cells.

The O subgroup of Forkhead box proteins (FoxO) might play an important role in cell growth, proliferation, differentiation, longevity, metabolism and tumor development (13-15, 38). The activity of FoxO

is controlled by post-translational modifications, including phosphorylation, acetylation and ubiquitination (12). It is a target for PI3K/Akt to induce tumorigenicity through serine/threonine phosphorylation and nuclear exclusion. Cellular stresses such as oxidative stimuli lead to FoxO acetylation causing an inhibition of FoxO activity (12-15, 39).

SIRT1, a member of sirtuin family is known to be regulator of lifespan extension. David et al. suggested that deacetylation of FoxO by SIRT1 increases its nuclear retention time and thus increases transcriptional activity (27). SIRT1 acts as one of the critical regulator of FoxO transcription in response to cellular stress via its role of NAD-dependent deacetylation (28). On the other hand, Motta et al. insisted that SIRT1 may down-regulate and repress forkhead factor including FoxO1 and FoxO4 as well as FoxO3a by destabilizing the protein, decreasing its DNA binding activity, or changing protein/protein interaction (17). They demonstrated counteraction of SIRT1 on p300-mediated activation of FoxO3a. In addition, Yang et al. asserted that FHL2 interacts with FoxO1 and invalidates transcriptional activity of FoxO1 through deacetylation mediated by SIRT1 in prostate cancer cells (40). Although there is a conflict of several opinions about

SIRT1 and FoxO1 regulation mechanism like this, recent studies showed SIRT1 as an assistor of FoxO activation. SIRT1-mediated deacetylation blocks FoxO inhibition introduced from acetylation and thereby prolongs FoxO-dependent transcription of subnuclear stress-regulating genes. Through this mechanism, SIRT1 is thought to be able to promote cellular survival and increase lifespan (41, 42).

In TAMR-MCF-7 cells not only SIRT1 but phosphorylated Akt levels are also increased. Activation of Akt phosphorylates FoxO1, subsequently incapacitates its transcriptional activity, which may have influence on MRP2 expression. However, TARM cells retain highly expressed FoxO1 not to have of MRP2. Our previous data can be a clue to solve this contradiction. PI3-kinase pathway plays a key role in controlling the PXR activity and PXR is thought to be an important transcription factor for MRP2 induction. When PI3K/Akt specific inhibitor LY294002 was treated in TAMR-MCF-7 cells, PXR reporter activities and MRP2 protein levels were significantly inhibited implying the role of PXR-mediated Akt in TAMR-MCF-7 cells. This action is unlikely to be dependent on FoxO1 action because LY294002 treatment could not change the FoxO1 protein expression.

Although MCF-7/ADR cells showed lower SIRT1 enzyme

activity leading to conclusion that other mechanism such as PI3K/Akt might be related with FoxO1 up-regulation, *MDR1* gene itself was also transactivated by SIRT1.

When SIRT1 was co-transfected with FoxO1, MRP2 and MDR1 transcriptional activities were significantly increased, while each one also contributes to induce MRP2 and MDR1 transactivation. Furthermore, this elevation caused by SIRT1 was reversed by FoxO1 suppression. Well-known SIRT1 inhibitor nicotinamide reduced the protein level and promoter activity of FoxO1 and MRP2 in our present study.

Recently some natural compounds are screened targeting SIRT1. In an effort to discover regulators of human SIRT1 enzymes, we performed a screen of several compounds. Out of 400 compounds from screening result of Dr. Oh, CPP342 and CPP343 showed remarkably potent effect. MDR1 and MRP2 levels were drastically reduced by treatment of both of them. CPP343 seems to have more highly efficacy than CPP342. FoxO1 and FoxO3 levels were also measured after treating CPP343. FoxO1 was significantly down-regulated by CPP343 and it influenced on MDR1 and MRP2 expression. Because there has been known to be complicated mechanism of resistance in

TAMR-MCF-7 cells, proper recover of drug responsiveness only by SIRT1 inhibition seems to be insufficient in this cell line. Hence an effort to search for more detail mechanisms of this phenomenon in TAMR-MCF-7 cells is needed. In case of MCF-7/ADR cells, SIRT1 inhibition is well adapted. CPP343 didn't show any severe cytotoxicity but down-regulated MDR proteins. Decreasing effects of these proteins turned out to be due to FoxO1 inhibition as shown in the result of reduced FHRE reporter activity by CPP343 treatment. Moreover, increased doxorubicin cellular uptake and doxorubicin-induced apoptosis results after treating with CPP343 are alluding to the possibility of CPP343 as an effector to reverse MDR proteins. Even if more studies on establishment of regulation mechanism of CPP343 by investigating other potential molecular target such PI3K/Akt are demanded, we herein affirmed that SIRT1 inhibitors have influence on FoxO1 activation and subsequently affect MDR transporters.

Overall, this study showed that FoxO1 positively regulates MRP2, and SIRT1 plays an important role in strengthening those effects. These are worthy of attention because it could be a new therapeutic target to overcome the chemoresistance cancers.

5. References

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6. Figures and Legends

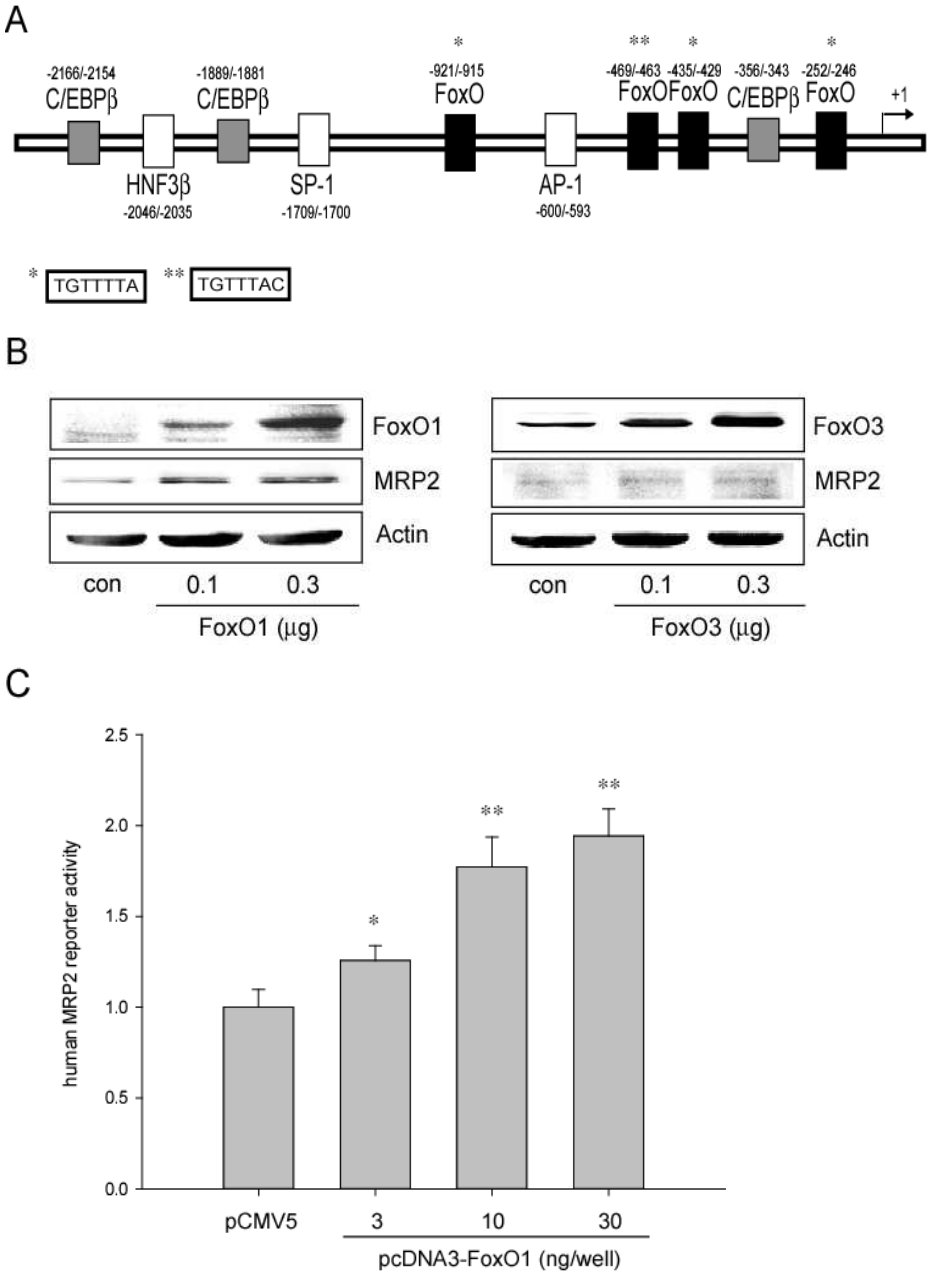
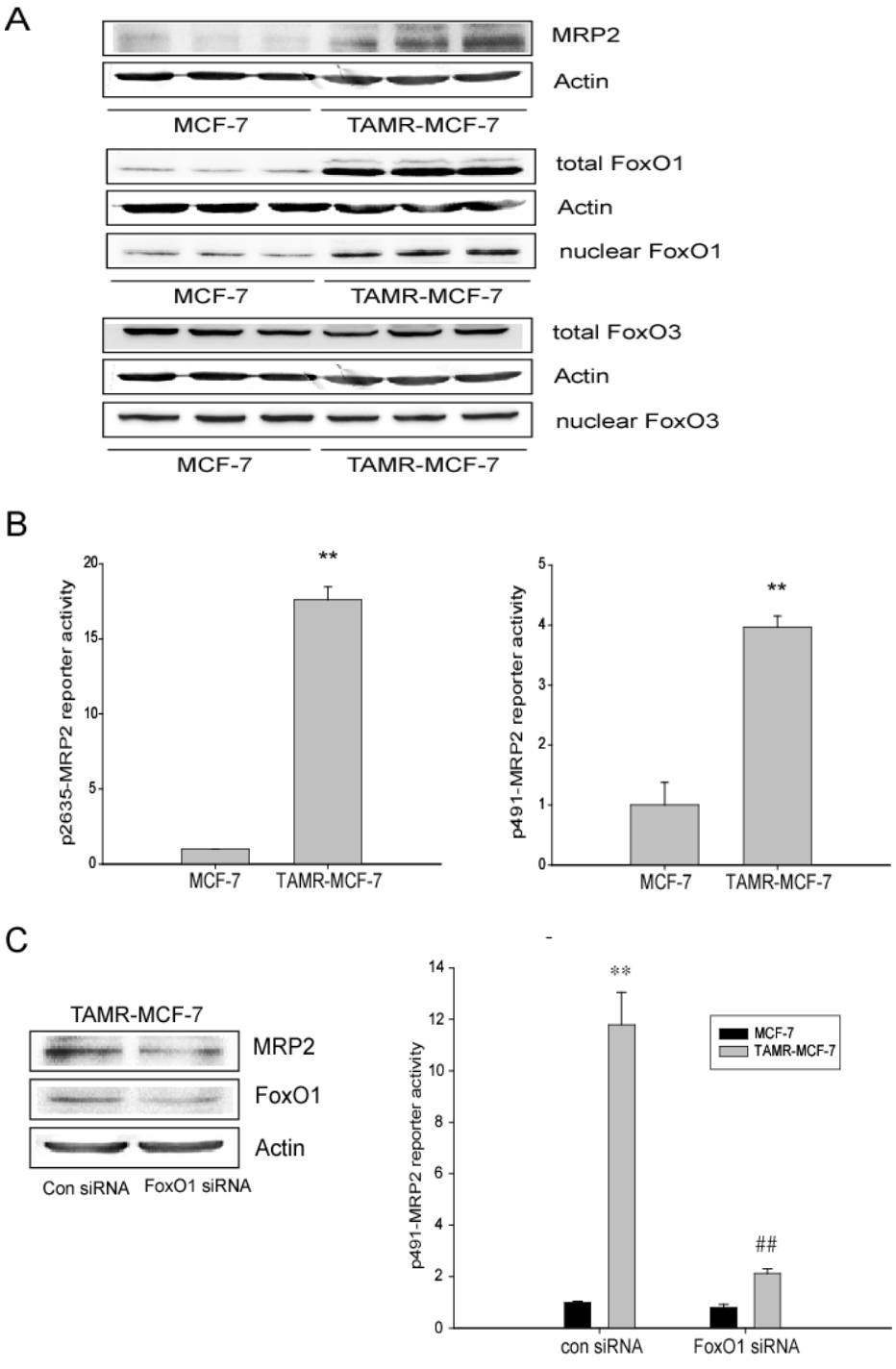


Figure 1. Regulation of MRP2 by FoxO1. (A) Putative binding sites to FoxO in the proximal promoter region of human *MRP2* gene. The predicted binding sites for transactivation factors are demonstrated. The numbering is relative to the major transcription initiation site, which is marked by an arrow. The asterisk (∗, ∗∗) indicates putative FoxO binding sites based on highly conserved sequence of Forkhead protein. (B) Induction of MRP2 proteins by FoxO overexpression. MCF-7 cells were cultured in transfection optimized medium with pcDNA3-FoxO1 or pcDNA3-FoxO3 for 6 h and in serum deprived medium for 18 h. Cell lysates were subjected to immunoblotting analysis with anti-MRP2 antibody. Equal loading of proteins was verified by actin immunoblotting. (C) FoxO1 up-regulates MRP2 transcriptional activity in MCF-7 cells. MCF-7 cells were co-transfected with a p2635-human MRP2 reporter construct and pcDNA3-FoxO1 in a concentration dependent manner (3-30 ng), followed by reporter assays. The data represents the mean ± S.D. of 3 separate samples (significant versus the control, ∗$p<0.05$; ∗∗$p<0.01$; control level = 1)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MCF-7
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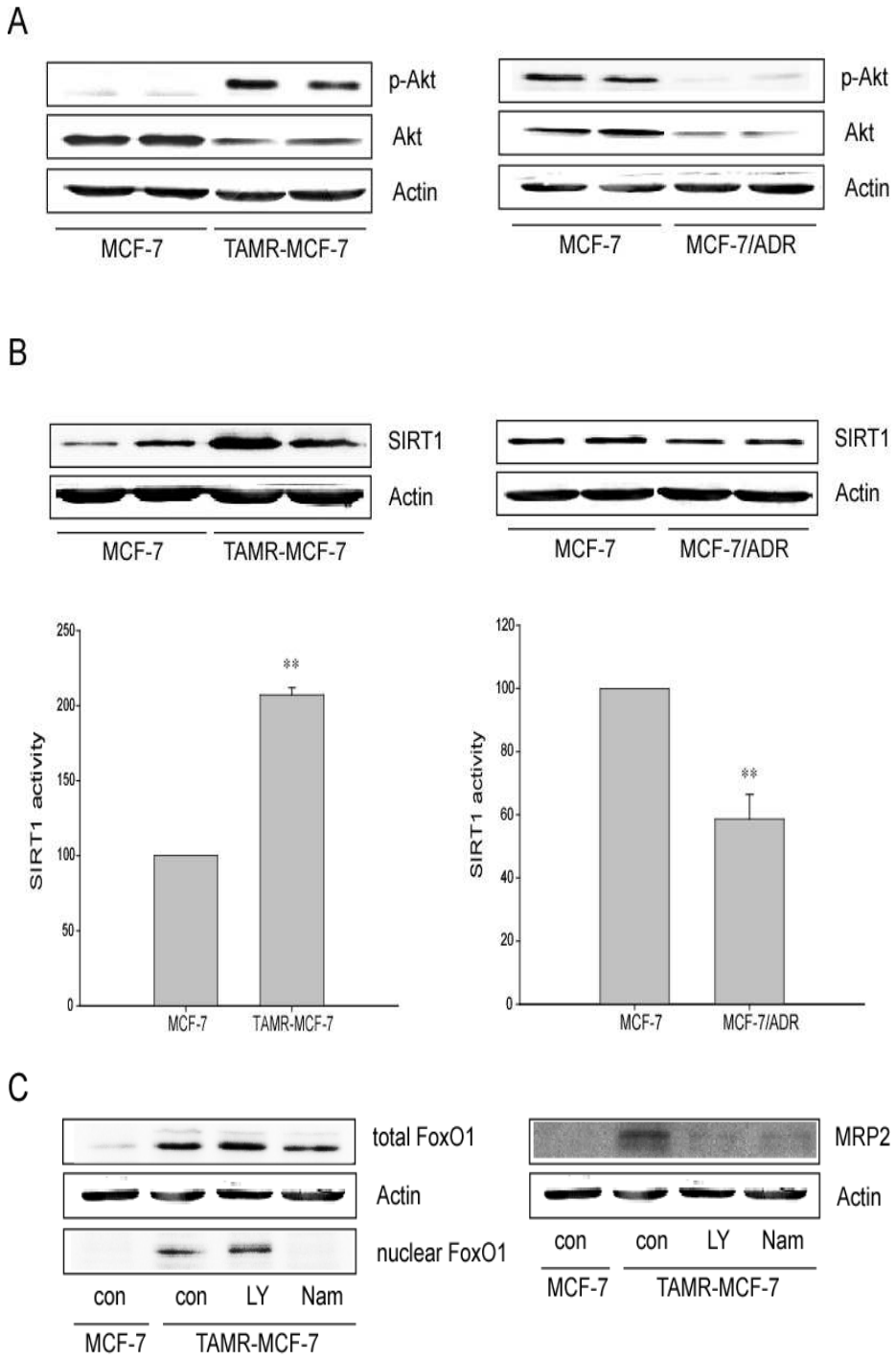
FoxO1 siRNA

Figure 2. Activation of MRP2 in TAMR-MCF-7 cells. (A) Immunoblot analysis of MRP2, FoxO1 and FoxO3 in the total cell lysates or nuclear fractions. A representative immunoblot shows MRP2 protein in both MCF-7 and TAMR-MCF-7 cells serum-deprived for 24 h (upper panel). Nuclear fractions and total cell lysates were also obtained from both MCF-7 and TAMR-MCF-7 cells in same conditions, and FoxO1 (middle panel) and FoxO3 (lower panel) in each fraction were immunoblotted with the respective antibody. (B) Reporter activities of deletion mutant human MRP2 promoters in MCF-7 and TAMR-MCF-7 cells. Each cell type was transiently transfected with p2635-MRP2-Luc (left panel) or p491-MRP2-Luc (right panel) plasmid. Dual luciferase reporter assays were performed on the lysed cells co-transfected with pMRP2-Luc plasmid (firefly lucifefase) and phRL-SV (hRenilla luciferase) (a ratio of 100:1) 18 h after transfection. Reporter gene activation was calculated as a relative ratio of firefly luciferase to hRenilla luciferase activity. Data represent means ± SD with 6 different samples (significant versus the control, $**p<0.01$; control level = 1). (C) Inhibition of MRP2 protein expression and transcriptional activity by FoxO1 suppression. MRP2 and FoxO1 levels were determined by immunoblotting in TAMR-MCF-7 cells transfected

with FoxO1 siRNA (60 pmole) or control siRNA (60 pmole) (left panel). MCF-7 and TAMR-MCF-7 cells were co-transfected with p2635-MRP2-Luc in combination with FoxO1 siRNA (20 pmole) or control siRNA (20 pmole) (right panel). Data represent the means ± SD with 6 separate samples (significant when compared with MCF-7 cells transfected with con siRNA, **p<0.01; significant when compared with TAMR-MCF-7 cells transfected with con siRNA, ##p<0.01; control level = 1).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TAMR-MCF-7
MRP2
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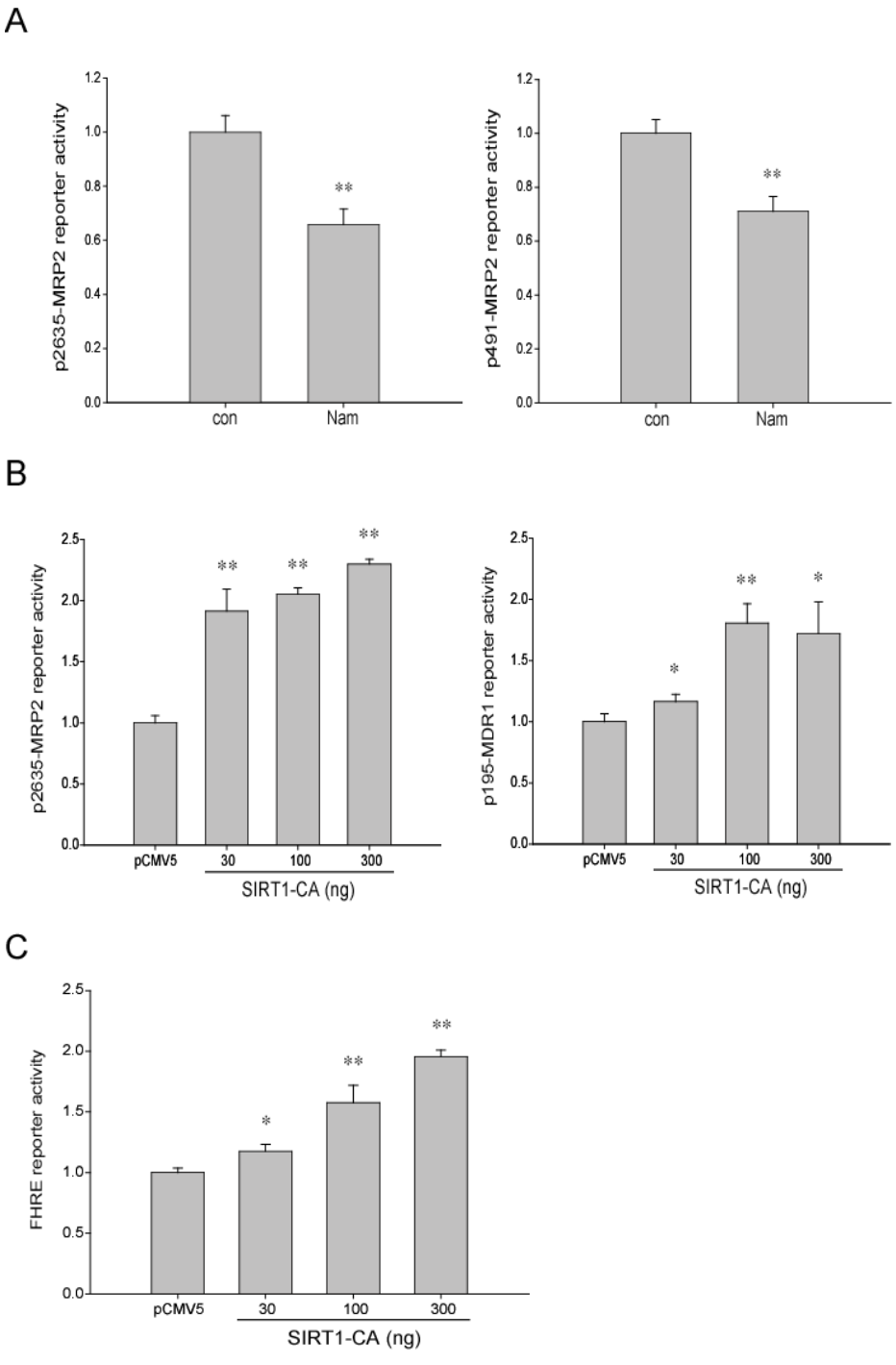
MCF-7
TAMR-MCF-7

Figure 3. The factors related with FoxO1 regulation mechanism. (A) Akt and phosphorylated Akt levels were detected in TAMR-MCF-7 cells (left panel) and MCF-7/ADR cells (right panel) compared with MCF-7 cells by immunoblotting. (B) SIRT1 protein expression in TAMR-MCF-7 cells (upper, left panel) and MCF-7/ADR cells (upper, right panel). SIRT1 activities were measured by Fluor de Lys Fluorescence assay. Cell lysates of TAMR-MCF-7 cells (lower, left panel) and MCF-7/ADR cells (lower, right panel) were obtained from the cells cultured in serum deprived medium for 24 h. Instead of SIRT1 enzyme, cell lysates were used to detect the relative activity of SIRT1. Data represents means ± SD with 3 different samples (significant versus the control MCF-7 cells, $**p<0.01$; control level = 1) (C) The effects of PI3-kinase inhibitor and SIRT1 inhibitor on the expression of FoxO1 and MRP2 in TAMR-MCF-7 cells. Expression of FoxO1 and MRP2 was measured in TMAR-MCF-7 cells treated with PI3-kinase inhibitor (LY; LY294002 20 μM) and SIRT1 inhibitor (Nam; nicotinamide 1 mM) for 24 h.


A
p2635-MRP2 reporter activity
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p491-MRP2 reporter activity
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SIRT1-CA (ng)
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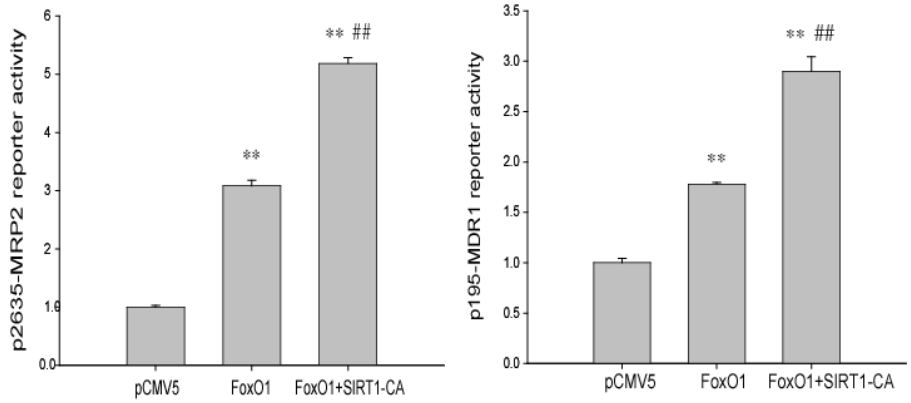
SIRT1-CA (ng)

Figure 4. Requirements of SIRT1 to induce FoxO1-mediated multidrug resistant proteins. (A) Inhibition of p2635-MRP2 and p491-MRP2 reporter gene activation in TAMR-MCF-7 cells by SIRT1 suppressions. TAMR-MCF-7 cells were treated Nam (nicotinamide; 1 mM), 10 min after transfection of cells with two types of MRP2 reporter plasmids and then the luciferase activity was measured 18 h after inhibitors treatment. Data represent the means ± SD of 3 different samples (significant when compared with control treated cells, $**p<0.01$; control level = 1). (B) Transactivation of MRP2 and MDR1 by SIRT1. Reporter activity of p2635-MRP2-Luc (left panel) and p195-MDR1-Luc (right panel) was measured in MCF-7 cells transiently co-transfected with SIRT1-constitutive active plasmid (30-300 ng) or pCMV5 vector. Data represents means ± SD with 3 different samples (significant versus the control, $*p<0.05$; $**p<0.01$; control level = 1). (C) Increased transcriptional activity of FHRE promoter by SIRT1. MCF-7 cells transiently co-transfected with FHRE reporter plasmid in combination with SIRT1-constitutive active plasmid (30-300 ng) or pCMV5 vector. Data represent the means ± SD of 3 different samples (significant versus the control, $*p<0.05$; $**p<0.01$; control level = 1).

A


p2635-MRP2 reporter activity
** ##
**
pCMV5
FoxO1
FoxO1+SIRT1-CA
p195-MDR1 reporter activity
** ##
**
pCMV5
FoxO1
FoxO1+SIRT1-CA

B

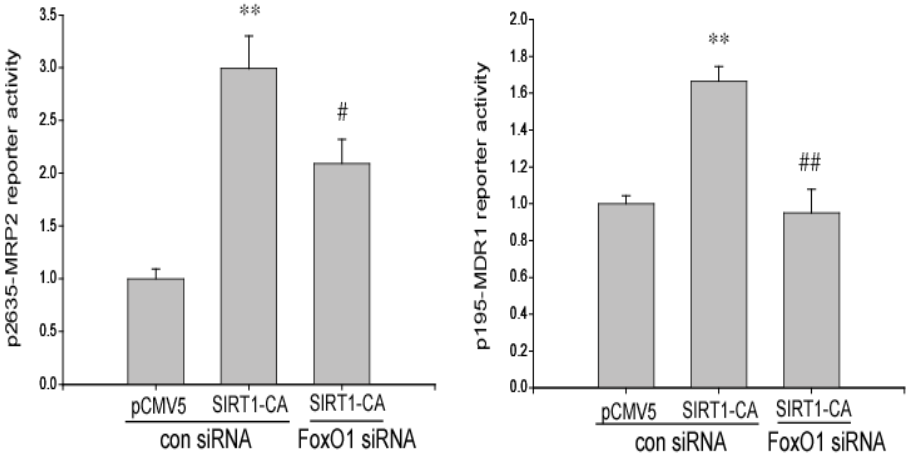
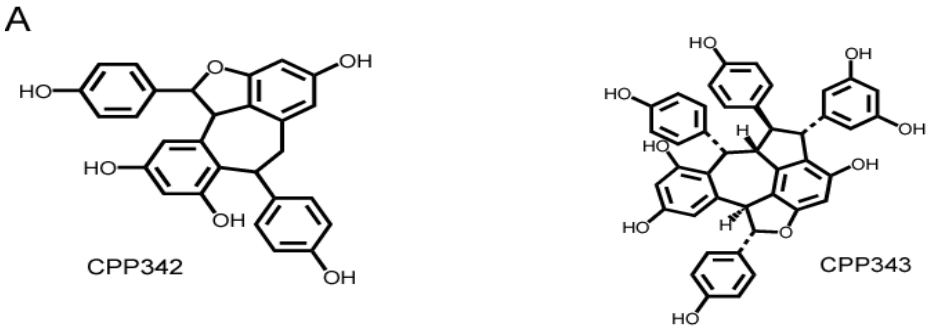
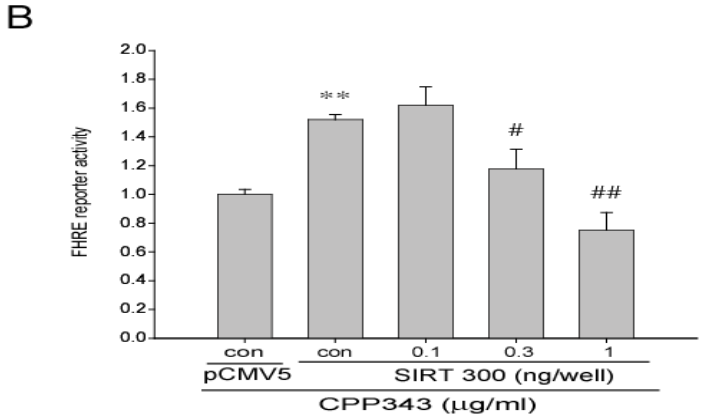
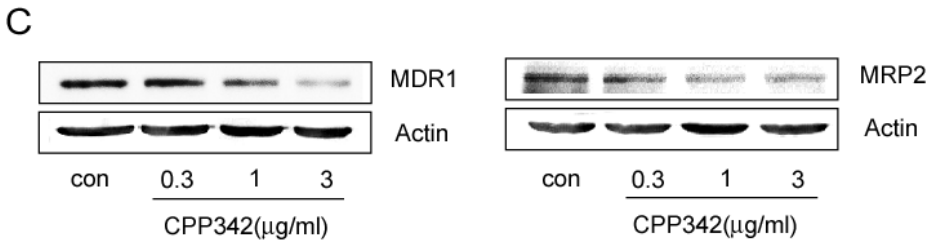

p2635-MRP2 reporter activity
**
#
pCMV5
SIRT1-CA
SIRT1-CA
con siRNA
FoxO1 siRNA
p195-MDR1 reporter activity
**
##
pCMV5
SIRT1-CA
SIRT1-CA
con siRNA
FoxO1 siRNA

Figure 5. The complementary effects of FoxO1 and SIRT1. (A) Up-regulation of MRP2 and MDR1 transactivation by FoxO1 and/or SIRT1. MCF-7 cells were co-transfected with pcDNA3-FoxO1 (30 ng), constitutive active form of SIRT1 (300 ng) plasmid and both of them with p2635-MRP2 (left panel) or p195-MDR1 (right panel) reporter plasmid and then the luciferase activities were measured 18 h after transfection. Data represent the means ± SD of 3 different samples (significant as compared to pCMV5-transfected MCF-7 cells, **p<0.01; significant as compared to FoxO1-trasnfected MCF-7 cells, ##<0.01; control level = 1). (B) Reversion of SIRT1 induced MRP2 and MDR1 transactivation by FoxO1 suppression. p2635-MRP2 reporter activities were measured in MCF-7 cells co-transfected with pCMV5 or SIRT1 (300 ng) in combination with FoxO1 siRNA (left panel). p195-MDR1 reporter activities were also measured in the same way (right panel). Data represents means ± SD with 3 different samples (significant when compared with control siRNA and pCMV5-transfected MCF-7 cells, **p<0.01; significant when compared with control siRNA and SIRT1-transfected MCF-7 cells, #p<0.05; ##p<0.01; control level = 1)


A
CPP342
CPP343


B
FHRE reporter activity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
#
##
con
con
0.1
0.3
1
pCMV5
SIRT 300 (ng/well)
CPP343 (μg/ml)


C
MDR1
Actin
con
0.3
1
3
CPP342(μg/ml)
MRP2
Actin
con
0.3
1
3
CPP342(μg/ml)

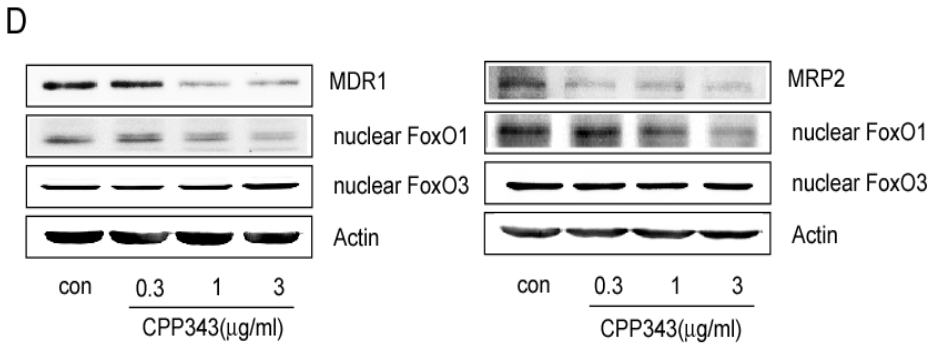

D
MDR1
nuclear FoxO1
nuclear FoxO3
Actin
con
0.3
1
3
CPP343(μg/ml)
MRP2
nuclear FoxO1
nuclear FoxO3
Actin
con
0.3
1
3
CPP343(μg/ml)

Figure 6. Effects of SIRT1-regulatory natural compounds, CPP342 and CPP343 on FoxO1-mediated MRP2 expressions. (A) Schematic of CPP342 and CPP343 structure. (B) Inhibitory effect of CPP343 on SIRT1-induced FHRE transactivation. MCF-7 cells were transfected with FHRE reporter plasmid together with pCMV5 or SIRT1 (300 ng). Six hours after transfection, the cells were either maintained as controls or were treated with CPP343 (0.1-1 μg/ml) for further 18 h. Data represent the means ± SD of 3 different samples (significant as compared to pCMV5-transfected MCF-7 cells, ∗∗$p<0.01$; significant as compared to SIRT1-trasnfected and untreated control MCF-7 cells, #$p<0.05$; ##<0.01; control level = 1). (C) Effect of CPP342 on MDR1 and MRP2 proteins. MCF-7/ADR cells (upper panel) and TAMR-MCF-7 cells were incubated with gradually increased concentrations (0.3-3 μg/ml) of CPP342 for 24 h and expression of MDR1 and MRP2 levels were measured by immunoblotting respectively. (D) Effect of CPP343 on MDR1 and MRP2 proteins. In addition, nuclear level of FoxO1 and FoxO3 was also measured. Total cell lysates and nuclear fractions of MCF-7/ADR cells and TAMR-MCF-7 cells were taken after treating CPP343 (0.3-3 μg/ml) for 24 h.

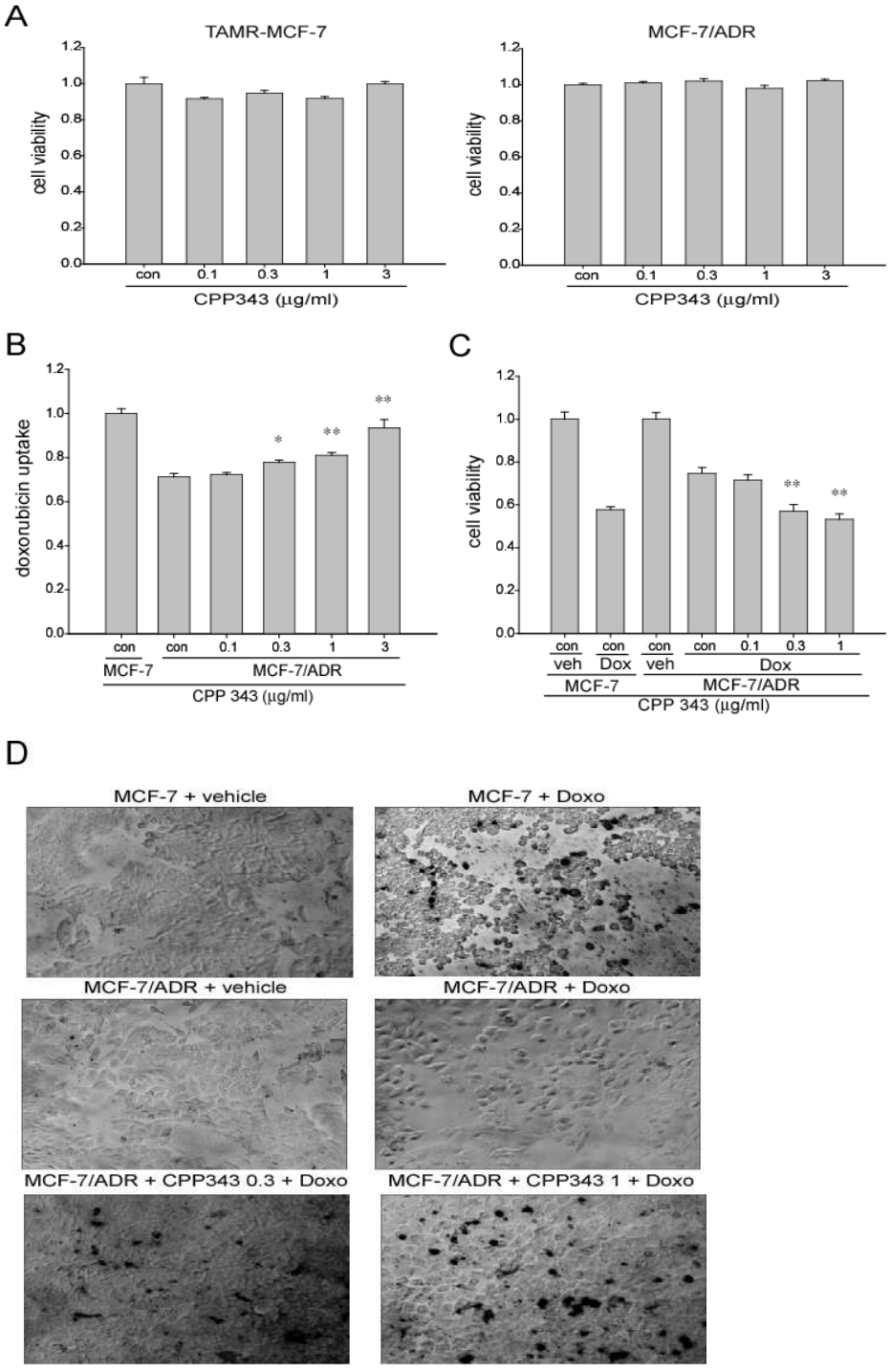

A
TAMR-MCF-7
MCF-7/ADR
cell viability
1.2
1.0
0.8
0.6
0.4
0.2
0.0
con
0.1
0.3
1
3
CPP343 (μg/ml)
B
doxorubicin uptake
*
**
**
MCF-7
MCF-7/ADR
CPP 343 (μg/ml)
C
cell viability
**
**
con
veh
Dox
Dox
MCF-7
MCF-7/ADR
CPP 343 (μg/ml)
D
MCF-7 + vehicle
MCF-7 + Doxo
MCF-7/ADR + vehicle
MCF-7/ADR + Doxo
MCF-7/ADR + CPP343 0.3 + Doxo
MCF-7/ADR + CPP343 1 + Doxo

Figure 7. Overcome of the resistance to doxorubicin in MCF-7/ADR cells treated with CPP343. (A) Cell viability after treating with CPP343. After incubation of TAMR-MCF-7 (left panel) and MCF-7/ADR (right panel) cells with or without CPP343 (0.1-3 μg/ml) for 24 h, cell viabilities were determined using MTT assays. Data represent the means ± SD of 14 different samples (control level = 1). (B) Doxorubicin cellular uptake. After incubation of MCF-7 and MCF-7/ADR cells with or without CPP343 (0.1-3 μg/ml) for 24 h, doxorubicin (30 μM) was treated for 60 min. Fluorescence intensities of doxorubicin retained in lysates of MCF-7 and MCF-7/ADR were measured using the excitation and emission wavelengths of 470 and 590 nm, respectively. The values were divided by total protein content of each sample. Data represent the means ± SD of 3 different samples (significant versus the untreated MCF-7/ADR cells, $*p<0.05$; $**p<0.01$; control level = 1). (C) Cell viability after treating with doxorubicin with or without CPP343 treatment. Doxorubicin (30 μM) was treated 24 h after exposure of MCF-7 and MCF-7/ADR cells with or without CPP343 (0.1-1 μg/ml). Cell viabilities were determined by crystal violet assays. Data represent the means ± SD of 14 different samples (significant versus the doxorubicin-treated and CPP343 control

MCF-7/ADR cells, **p<0.01; control level = 1). (D) Representative photographs of TUNEL assays on cells cultured with or without 30 μM doxorubicin for 24 h. MCF-7/ADR cells were pretreated with CPP343 (0.3-1 μg/ml) 24 h before doxorubicin exposure. Results were confirmed by multiple experiments.

저작물 이용 허락서

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논문제목	한글 : 타목시펜 저항성 유방암 세포에서 MRP2 를 발현 시키는 FoxO1 활성에 대한 SIRT1 의 역할 영어 : Requirement of SIRT1-mediated FoxO1 activation for Multidrug Resistance-Associated Protein 2 expression in Tamoxifen resistant breast cancer cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2009 년 2 월 25 일

저작자: 조 경 빈 (서명 또는 인)

조선대학교 총장 귀하

감사의 글

아직도 부족한 논문에 자꾸만 손이 가서 읽고, 또 읽고, 또 고치기를 반복하다가 드디어 마음 먹고 이 곳에 다다랐습니다. 섣불리 도전했던 지난 학기에 이어 졸업 준비를 먼저 시작했음에도 불구하고 여전히 모자람을 많이 느끼고 있습니다. 한편으로 생각해 보면 이런 나 자신이 이렇게 구색을 갖추어서나마 논문을 낼 수 있게 되기까지는 정말 많은 사람의 도움이 있었을 것이라는 생각을 다시금 해보게 됩니다. 그런 만큼, 다소 허술한 점이 있더라도 논문에 실려 있는 고마운 사람들의 무게만은 감히 재어 볼 수도 없을 것 같습니다. 그런데도 이런 고마움을 표현하기란 왜 이렇게 쑥스러운지 영어로 쓰는 논문 못지 않게 쉽사리 말을 이어 나가기 벅찹니다. 고마움과 죄송한 마음이 교차하여 잠깐씩 숨을 고르며 천천히 한 분, 한 분을 떠올려야겠습니다. 이 글을 읽어 주시는 모든 사람들이 감사의 대상이 될 것이고, 그 분들이 이 글을 읽으며 제 진심을 알아주는 것에도 의의가 있지만 저 자신 역시 앞으로 그 고마움을 잊지 않고 더욱 열심히 살아가기 위해 이 한 글자, 한 글자를 정성스럽게 채우려 합니다.

격려와 지도를 아끼지 않고 늘 열성적인 모습으로 저를 포함한 많은 학생들의 표본이 되어 주신 약학과 교수님들께 감사합니다. 지도 교수님인 강건욱 교수님께는 가장 먼저 감사의 인사 올립니다. 나태해질 때마다 교수님께서 해 주신 말씀들과 즐겁게 연구하시는 모습을 보고 마음가짐을 다잡을 수 있었습니다. 학부생 시절 약물학 실험 노트의

고찰 밑에 달아주신 잘했다는 교수님의 답글 하나로 흥미가 생겨 실험실에 첫발을 들여 놓게 되었고 연구의 꿈을 키우게 되었습니다. 잘하고 잘못함을 엄격하게 구분하여 적절하게 칭찬과 훈계를 해 주셔서 즐거움과 반성하는 마음으로 제가 조금씩 발전하고 진지하게 실험에 임할 수 있게 된 것 같습니다. 위로와 격려의 말씀 건네며 힘을 주신 한효경 교수님, 정혜광 교수님, 최후균 교수님, 실험에 대한 새로운 지식을 깨닫게 해주고 이 논문에 결정적인 도움을 주신 최홍석 교수님과 오원근 교수님께도 이루 말할 수 없는 감사의 마음을 전합니다.

가끔 아옹다옹 하기도 했지만 무려 2년 동안 함께하며 잘 챙겨주고 많이 알려 주었던 실험실 동기인 상은 오빠와 미라에게 고맙습니다. 또한 이번에 같이 졸업하게 된 Yuba씨와 네팔에 있는 그리운 Sindhu씨, Shreeya에게도 전합니다 'I express my heartfelt gratitude to Yuba, Sindhu, Shreeya. I really miss you!'. 헤어질 것을 생각하면 섭섭하지만 모두 하고자 하는 바람 이루어 멋진 모습으로 다시 만나기를 진심으로 기원합니다. 언제나 편안하게 대해주며 실험에 관한 정보는 물론, 귀여운 현민이 이야기와 맛있는 이야기로 활력을 불러준 정우 오빠와 옥이 언니에게도 고마움을 전합니다. 새로운 베트남 학생인 Phuong이 실험실에서 즐겁게 생활하며 열심히 공부하길 바랍니다. 선배로써 많은 것을 알려주고 실험에 대해 많은 지도와 격려 아끼지 않았던 창엽 오빠와 상희 언니, 먼 곳에서 찾아와 준 정용 오빠와 진원 오빠에게도 감사합니다. 미국 학회 & 라스베가스 동지인 생화학실 나연이, 약전실의 온갖 지식인인 영빈이와 여유로움이 부러운 친구

운정이, 물리약학실 명학 오빠, 형주 오빠, 영진, 선자씨, 선임씨, 위생약학실 선생님들에게도 이것저것 많은 도움과 신세를 졌습니다. 그리고 늘 응원해 주는 친구들과 선배, 후배들에게도 정말 고마움을 느끼고 있습니다. 모두 더욱더 번창하길 진심으로 기원합니다. 김옥자 선생님을 비롯해 대학원 교학팀과 행정실에서 신경 써 주신 선생님들, 늦은 시간까지 고생하시는 수위 아저씨들과, 엄청난 양의 폐기물과 쓰레기를 깨끗하게 치워주시는 아주머니들께도 미처 전하지 못했던 감사 인사 드립니다.

마지막으로 철부지 막내딸을 믿고 아껴 주시고 언제나 걱정하고 챙겨 주시는 엄마, 아빠와 늘 격려하고 도와주는 언니에게 어떻게 해도 표현하지 못할 만큼 넘치도록 사랑하고 감사한다고 꼭 말하고 싶습니다. 그리고 그 누구보다도 미안한 마음뿐입니다. 바쁘다는 핑계로 소홀히 했던 일들 반성하고 가족들에게 더 잘하는 멋진 딸과 동생이 되겠습니다. 저를 위해 기도해 주시는 할아버지, 할머니, 친척들에게도 감사하고 더 발전하는 모습 보여드릴 수 있도록 열심히 하겠습니다.

이제 겨우 제 손길이 스쳐간 논문 한 권을 냈을 뿐이지만 저를 응원해 주고 도와 주셨던 많은 분들을 한 명씩 떠올려 보니 포만감처럼 든든하게 마음이 차오르는 것을 느낄 수 있습니다. 때로는 기쁜 일도, 슬픈 일도, 희망도, 좌절도 번갈아 가며 찾아 오겠지만 이 든든한 기분을 생각하며 어떤 일이든 열심히 임하며 살아가겠습니다. 다른 사람에게 도움을 줄 수 있는 멋진 사람이 되어야겠다는 저의 꿈을 포기하지 않고 꼭 이루겠습니다. 감사합니다.