Effect of \( Y_6 \), an epigallocatechin gallate derivative, on reversing doxorubicin drug resistance in human hepatocellular carcinoma cells

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ABSTRACT

Cancer cells can acquire resistance to a wide variety of diverse and unrelated drugs, this phenomenon is termed multidrug resistance (MDR). Multidrug resistance has been an obstacle to the success of cancer chemotherapy. The present study investigated the reversal effect of \( Y_6 \), a new compound obtained by chemically modifying the structure of epigallocatechin-3-gallate (EGCG) extracted from green tea. \( Y_6 \) was proven to be effective in inhibiting cell proliferation and reversing drug resistance in doxorubicin (DOX) resistant human hepatocellular carcinoma cells (BEL-7404/DOX). BEL-7404/DOX cells were treated with either doxorubicin combination regimen (doxorubicin plus \( Y_6 \) or epigallocatechin-3-gallate or verapamil separately) or doxorubicin alone. The results showed that cell proliferation was inhibited and late cell apoptosis increased in the combination treatment group, especially in the group treated with doxorubicin plus \( Y_6 \). Further analysis revealed that the expressions of hypoxia-inducible factor-1\( \alpha \) and multidrug resistance 1/P-glycoprotein decreased at both messenger RNA and protein levels by treatments with combined drugs compared to doxorubicin alone. Our results indicated that \( Y_6 \) as a drug resistance reversal agent, increased the sensitivity of drug resistant cells to doxorubicin. The mechanisms of actions of \( Y_6 \) in reversal effect were associated with the decreased expression of hypoxia-inducible factor-1\( \alpha \) and multidrug resistance 1/P-glycoprotein.

INTRODUCTION

Liver cancer is a malignancy of high incidence and mortality. It is estimated that nearly 750,000 new cases and more than 690,000 cancer deaths occur annually worldwide. Unfortunately, half of these cases were estimated to occur in China [1]. Among the liver cancers, hepatocellular carcinoma (HCC) is the major histological subtype. Various anticancer drugs such as tamoxifen, octreotide, interferon, and interleukin-2 are used in the treatment of HCC; however, these drugs are not specific for HCC and demonstrate low treatment efficacy [2].
Multidrug resistance (MDR) to anticancer drugs is the most common cause of failure in cancer chemotherapy [3]. The mechanisms of resistance to anticancer drugs consist of changes of pharmacokinetic or tumor microenvironment, cancer cell-specific factors which include increased drug efflux or decreased drug influx, drug inactivation, drug target modification, and apoptosis evasion [4]. Of these mechanisms, the increased drug efflux/reduced drug accumulation in the tumor cell appears to be a very common mechanism of MDR. Hydrophobic drugs are expelled from cells by activation of energy-dependent transport systems [5, 6]. The first of these transporters to be identified was P-glycoprotein (P-gp), the product of the human MDR1 gene [7]. P-gp is a member of the large adenosine triphosphate (ATP)-binding cassette (ABC) family of proteins, also known as ABCB1. P-gp/ABCB1 has a molecular weight of 170 kDa and comprises of two nucleotide-binding domains (NBD1 and NBD2) and two transmembrane-binding domains (TMD1 and TMD2) [8, 9]. P-gp/ABCB1 utilizes energy from the hydrolysis of ATP to efflux drugs from intracellular to extracellular matrix, leading to decreased intracellular drug concentration [10]. Overexpression of P-gp/ABCB1 can produce MDR in cancer cells [11].

Over the past few decades, efforts were made to look for new compounds as resistance reversal agents to overcome MDR in tumor cells. Verapamil was one of the first generation of these MDR reversal agents [12]. But the effective concentration of verapamil in reversing MDR in vitro was too high to be achieved in vivo [13]. The dose of verapamil required was much larger than the clinically safe dose, resulting in toxic reactions in almost all patients. Although second and third generations of reversal agents were explored, they were inhibited respectively by P450 3A4 enzymes and by anticancer drugs [14–16]. In order to obtain more effective and safer resistance reversal agents, some natural products and their derivatives have been considered for use in combination with anticancer drugs [17].

Epigallocatechin-3-gallate (EGCG) is one of the MDR reversal modulators (Figure 1A) [18]. It is the most abundant catechin in green tea polyphenols. A previous study revealed that EGCG could significantly inhibit the proliferation of human HCC cell BEL-7404/DOX in vitro and the tumor growth of the xenograft mouse model when it was administered at lower doses with doxorubicin, compared to treatment with doxorubicin alone [19]. However, many phenolic hydroxyl groups are included in the structure of EGCG, which makes the compound unstable due to rapid oxidation, low lipid solubility, low bioavailability, and short duration of action. Therefore, its application became limited [20]. Y$_6$ is an ethylation product of EGCG with strong stability (Figure 1B). The above limitations were overcome in Y$_6$, because most of the phenolic hydroxyl groups were replaced by ethyl groups. In the present study, we evaluate the potential effect of Y$_6$ as a reversal agent that specifically reverses ABC transporter-mediated MDR in vitro.

RESULTS

Y$_6$ improved the chemosensitivity of BEL-7404/DOX cells to doxorubicin treatment

HCC cell lines (parental BEL-7404 and doxorubicin-selected resistant BEL-7404/DOX cells) were used to examine the cytotoxicity of doxorubicin, EGCG, and Y$_6$ on cell proliferation using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. As shown in Figure 2, higher concentration of doxorubicin is required to inhibit the same ratio of BEL-7404/DOX cell proliferation than that required in BEL-7404 cells. The IC$_{50}$ value (44.14 μM) for doxorubicin in the BEL-7404/DOX cells was 29.2 times higher than that (1.51 μM) in the parental BEL-7404 cells (Table 1). The IC$_{50}$ values of Y$_6$ and EGCG in this pair of cell groups were > 50 μM. The concentrations for combined use with doxorubicin were non-cytotoxic and below the IC$_{50}$ values (Figure 2).

The non-toxic doses of verapamil, EGCG, and Y$_6$ were individually combined with a series of doxorubicin concentrations, and were used to treat BEL-7404 cells and BEL-7404/DOX cells. Among them, verapamil, a known chemosensitizer, was used as the positive control inhibitor of P-gp in this experiment. The MTT assay results showed that the IC$_{50}$ values for doxorubicin was significantly reduced from 44.14 to 8.39 μM by verapamil, to 8.04 μM by EGCG, and to 5.74 μM and 4.36 μM by Y$_6$ (10 μM) and Y$_6$ (15 μM) respectively in the BEL-7404/DOX cells compared with doxorubicin alone, representing drug resistance folds decreasing from 29.2 to 5.6, 5.3, 3.8, and 2.9 respectively (Table 1). Combination groups were compared with the doxorubicin alone group, and the differences were statistically significant ($p < 0.05$). These results indicated that the capability of Y$_6$ in reversing drug resistance was higher than that of EGCG combined with doxorubicin ($p < 0.05$) (Table 1, Figure 2).

Y$_6$ induced apoptosis in BEL-7404/DOX cells

The induction of cell apoptosis is a common mechanism for many anti-tumor drugs. To examine whether Y$_6$ can induce cell apoptosis, we detected apoptotic cells in HCC BEL-7404/DOX cells treated with verapamil (10 μM), EGCG (10 μM), and Y$_6$ (10 and 15 μM) in combination with doxorubicin (10 μM) and compared with doxorubicin (10 μM) alone. The cells that treated with verapamil were served as the positive controls of P-gp inhibitors. BEL-7404/DOX cells were incubated in anoxic condition for 48 h, then subjected to Annexin V-FITC labeling and Propidium iodide (PI) staining as described in the Materials and Methods section. We used flow cytometry analysis to determine the apoptotic rate of BEL-7404/DOX cells treated with the drug combinations or doxorubicin alone. The results showed that after treatment with any drug combination, the
The number of cells increased in late apoptotic stage (Table 2, Figure 3). Only 12.17% of cells showed apoptotic signals when treated with doxorubicin alone, but the percentage increased to 17.91% with verapamil (10 μM), to 19.52% with EGCG (10 μM), to 27.89% with Y₆ (10 μM) and to 40.03% with Y₆ (15 μM). The differences were statistically significant when compared with the doxorubicin group (*p < 0.05). Furthermore, at the same concentration, Y₆ had a higher effect than verapamil and EGCG on the induction of apoptosis in the late apoptotic stage, and the differences were statistically significant (#p < 0.05). In addition, the higher the concentrations of Y₆, the more cell apoptosis in the BEL-7404/DOX cells.

Y₆ inhibited the expression of hypoxia-inducible factor-1α (hif-1α) and MDR1 mRNA in BEL-7404/DOX cells

Previous studies have shown that high MDR1-gene expression exists in BEL-7404/DOX cells compared with BEL-7404 cells [21, 22]. Therefore, differences in expressions of hif-1α and MDR1 genes were investigated by quantitative real time polymerase chain reaction (RT-PCR) analysis after drug treatment. The results showed that the expression of hif-1α was significantly lower in the combination drug therapy groups (doxorubicin plus EGCG, verapamil, or Y₆) than in the doxorubicin alone group. Expression of hif-1α was 0.90 in BEL-7404/DOX cells treated with doxorubicin (10 μM) alone, but it decreased to 0.65 with verapamil (10 μM), to 0.49 with EGCG (10 μM), to 0.19 with Y₆ (10 μM), and to 0.10 with Y₆ (15 μM). For the MDR1 gene, the expressions were significantly lower in the combination drug therapy groups (doxorubicin plus EGCG or Y₆, excluding verapamil) than that in the doxorubicin alone group. The expression of MDR1 was shown to be 0.71 in BEL-7404/DOX cells treated with doxorubicin (10 μM) alone, but it decreased to 0.39 with EGCG (10 μM), to 0.25 with Y₆ (10 μM), and to 0.19 with Y₆ (15 μM). The differences were statistically significant not only in hif-1α, but also in MDR1, when compared with the doxorubicin group (p < 0.05) (Table 3, Figure 4).

![Chemical structures of two monomers of catechin](image-url)

**Figure 1:** Chemical structures of two monomers of catechin.
These results indicated that treatment of Y₆ decreased the expression of hif-1α and MDR1 mRNA.

**Y₆ inhibited the expression of HIF-1α and P-gp proteins in BEL-7404/DOX cells**

Since PCR results showed that treatment with Y₆ inhibited the mRNA expressions of hif-1α and MDR1 genes compared with the doxorubicin alone group, we investigated whether the expressions of their encoded proteins HIF-1α and P-gp were also inhibited. The expressions of HIF-1α and P-gp proteins were examined by Western blotting analysis. The results showed that the expressions of HIF-1α and P-gp proteins were significantly decreased in the combination drug therapy groups (doxorubicin plus Y₆) compared to the doxorubicin alone therapy group (*p < 0.05 and #p < 0.05) (Figure 5).

### Table 2: The late stage apoptosis rates in BEL-7404/DOX cells (n = 3)

| Drugs (μM)          | Apoptosis rate (%) ± SD (μM) |
|---------------------|------------------------------|
| Control              | 5.67 ± 1.10                  |
| DOX 10 μM           | 12.17 ± 1.26                 |
| +VER 10 μM          | 17.91 ± 3.35*                |
| +EGCG 10 μM         | 19.52 ± 4.41*                |
| +Y₆ 10 μM           | 27.89 ± 2.53**               |
| +Y₆ 15 μM           | 40.03 ± 3.21**               |

* Values represent means ± SDs of at least three independent experiments performed in triplicate. a: Control group, no drugs were used; b: DOX group, 10 μM of DOX alone was used; c: 10 μM of DOX combined with 10 μM of VER, 10 μM of EGCG, 10 μM of Y₆, or 15 μM of Y₆ were used; (VER 10 μM+DOX 10 μM) group was used as the positive control; *p < 0.05 vs. the DOX alone group. **p < 0.05 vs. the (VER 10 μM +DOX 10 μM) group or (EGCG 10 μM +DOX 10 μM) group.
DISCUSSION

Nature is an endless source of organic compounds for development of new drugs including resistance reversal agents for cancer. EGCG, from the Chinese green tea, is the effective ingredient for reversing anti-cancer drug resistance. Alkylate modification is one way to remedy the EGCG’s defects of low lipid solubility and stability [23]. Previous studies have shown that the ethylated and propyl derivatives of EGCG had strong reversal effect, while EGCG-methylated derivatives had no effect on the MDR in hepatocellular carcinoma cells [24]. However, with the increase of carbon chain, the toxicity to normal hepatocyte is also increased. Therefore, the propyl derivatives were more appropriate to be the potential anticancer drugs [24]. We synthesized six different EGCG-ethylated derivatives. Among them, Y₆ had not only more stable features but also better lipid solubility when compared with EGCG and other EGCG-ethylated derivatives. In this study, we elucidated the effect and mechanism of Y₆ in reversing drug resistance in BEL-7404/DOX cells.

By combining non-cytotoxic concentrations of Y₆ (below the IC₅₀ values) with doxorubicin, we determined that Y₆ potentiated the doxorubicin cytotoxic effects on cancer cells. Our MTT assay result showed that Y₆ and EGCG could enhance the cytotoxicity of doxorubicin by reversing multidrug resistance in BEL-7404/DOX cells. In addition, flow cytometric analysis result revealed that Y₆ increased cell apoptosis in the late apoptotic stage. The reversal effects of Y₆ were higher than that of EGCG both in MTT assay and in flow cytometric analysis experiments. Our study demonstrated that Y₆ has excellent ability in increasing cell sensitivity to doxorubicin and inducing apoptotic cell death.

Hypoxia is a common feature in solid tumors and is generally associated with tumor chemo-resistance [25, 26]. Hypoxia may reduce tumor sensitivity to radiation therapy and chemotherapy by deprivation of the oxygen essential for the cytotoxic actions directly, and lead to increasing resistance by inducing proteomic and genomic changes indirectly [27]. In the study by Du Cetal, hypoxia was shown to enhance tumor development in HCC [28].

Table 3: The expression of hif-1α and MDR1 genes in BEL-7404/DOX cells (n = 3)

| Gene Names | 2-ΔΔCT ± SD a | Control | DOX(10)b | EGCG(10) + DOXC | VER(10) + DOXC | Y6(10) + DOXC | Y6(15) + DOXC |
|------------|----------------|---------|-----------|----------------|---------------|---------------|---------------|
| hif-1α     | ——             | 0.90 ± 0.07 | 0.49 ± 0.18 | 0.65 ± 0.13* | 0.19 ± 0.10* | 0.10 ± 0.06* |
| MDR1       | ——             | 0.71 ± 0.18 | 0.39 ± 0.12* | 0.46 ± 0.15* | 0.25 ± 0.09* | 0.19 ± 0.08* |

The expression of hif-1α and MDR1 genes were measured in BEL-7404/DOX cell by RT-qPCR analysis. a: Values represent means ± SDs of at least three independent experiments performed in triplicate; b:DOX group, 10 μM of DOX alone was used; c:10 μM of DOX combined with 10 μM of VER, 10 μM of EGCG, 10 μM of Y₆, or 15 μM of Y₆ were used; VER (10)+DOX (10) was used as the positive control; Control group, no drugs were used; *p < 0.05 vs. the DOX alone group on the hif-1α gene; #p < 0.05 vs. the DOX alone group on the MDR1 gene; 'p > 0.05 vs. the DOX alone group on the MDR1 gene.

Figure 3: Apoptosis was measured in BEL-7404/DOX cell line. (A) Apoptotic cells as a result of Y₆, EGCG, and verapamil treatment were quantified by the Annexin V/PI assay. Cells were stained using an Annexin V/PI staining kit and then detected by flow cytometry. (B) *p < 0.05 vs. the doxorubicin alone group. 'p < 0.05 vs. the (verapamil 10 μM+doxorubicin 10 μM) group or (EGCG 10 μM+doxorubicin 10 μM) group. Experiments were performed for three independent times.
In the experiment by Yu G et al, the chemosensitivity to 5-FU or cisplatin in HBx-HepG2 cells was measured by MTT assay in different hypoxic (5% O₂, 3% O₂, or 1% O₂) conditions. The results revealed that under hypoxic conditions, particularly at 1% O₂, the cancer killing effects of 5-FU or cisplatin were prominently abrogated [29]. Therefore our experiment adopted the hypoxic condition of 1% O₂ to imitate the hypoxic environment of solid tumors in vivo. HIF-1 is a central component of hypoxic adaptation. It has been presented as an important mediator of hypoxia-regulated gene expression and can activate the transcription of genes that are important to cell viability, invasion, metastasis, and angiogenesis [30–32]. Hypoxia may significantly increase the protein expression level of HIF-1α [29]. The activation of HIF-1 is closely related to the increased expression of MDR1 gene and its product P-gp protein [33–38]. P-gp protein was the first transporter to be identified that associated to MDR. It was also the most representative and widely studied in reversing resistance. Previous study showed that expression of HIF-1α and MDR1/P-gp could be regarded as a predictive marker of chemotherapy resistance, and HIF-1α inhibition was use to reverse multidrug resistance by reducing expression of MDR1/P-gp [39]. Other studies revealed that the expressions of hif mRNA and its encoded protein were decreased after treatment with verapamil [40], but there was no decrease in the level of P-gp protein [41].

To investigate whether the effect of Y₆ on the reversal of resistance was associated with decreased P-gp expression via the HIF-1α-mediated pathway, we measured the mRNA expressions of hif-1α and MDR1 genes by quantitative RT-PCR analysis and expressions of their encoded proteins of HIF-1α and P-gp by Western blotting. The BEL-7404/DOX cells were pretreated in hypoxic condition before experiment. The results of our study revealed that expressions of hif-1α gene and its encoded protein were significantly down-regulated upon the treatment with doxorubicin plus Y₆. We also observed a significant decreased expression of MDR1 gene and its encoded protein P-gp in parallel with a decrease in HIF-1α expression in BEL-7404/DOX cells. The coincident decline of HIF-1α and MDR1/P-gp expression treated with Y₆ suggested a positive correlation between the two proteins. Y₆ were more potent in decreasing the expressions of HIF-1α and P-gp both in gene and in protein level than EGCG. The strong reversal effect of Y₆ was most likely due to the decline of P-gp transporter.

In conclusion, the present investigation suggested that Y₆ might reverse P-gp-mediated MDR. Y₆ in combination with doxorubicin could increase the cytotoxicity of doxorubicin in vitro. The mechanisms of Y₆ in reversing MDR are likely to be related to the HIF-1α-MDR1/P-gp pathway. Y₆, as a novel inhibitor resulting from the modification of natural product, may become an effective drug to overcome MDR in the treatment of hepatocellular carcinoma.

MATERIALS AND METHODS

Materials

EGCG (purity 97%) from green tea was obtained from Hangzhou Hetian biotechnology Co., Ltd. (Hangzhou, Zhejiang, China). Y₆ (purity 96.87%) was synthesized by our research group and was dissolved in dimethylsulfoxide (DMSO) to form a stock solution of...
10 mM and stored at −20°C. Verapamil hydrochloride was obtained from Shanghai Hefeng Pharmaceutical Co., Ltd. (Shanghai, China). Doxorubicin was purchased from Shanxi Pude Pharmaceutical Co., Ltd. (Datong, Shanxi, China). DMSO was purchased from Guangzhou Xingang Co., Ltd. (Guangzhou, Guangdong, China). 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide was purchased from Sigma (St. Louis, MO). An Annexin V-FITC/ Propidium iodide (PI) staining assay kit was obtained from Sony Biotechnology Inc. (San Jose, CA). Total Ribonucleic Acid (RNA) extraction reagent kit (RNAiso plus) and Prime Script™ RT reagent Kit with cDNA Eraser for Perfect Real Time were obtained from TAKARA Bio Inc. (Dalian, China). The anti-HIF-1α, anti-P-gp antibody, and anti-rabbit IgG (H+L) (Dy Light 680 Conjugate) were purchased from Cell Signaling Technology (Beverly, Massachusetts). β-actin polyclonal antibody was obtained from Bioworld Technology, Inc. (St. Louis Park, MN). RPMI-1640 medium was obtained from Hyclone Laboratories (Logan, UT), and fetal bovine serum (FBS) was from Hangzhou Sijiqing Co., Ltd. (Hangzhou, Zhejiang). Penicillin-Streptomycin Liquid (PS; 10,000 U/mL) and trypsin-EDTA Solution (containing 0.25% trypsin and 0.02% EDTA) were obtained from Beijing Solarbio Science&Technology Co., Ltd. (Beijing, China).

**Hypoxia cell culture**

Cells were cultured under normoxic condition (2% O₂, 5% CO₂, 74% N₂) for 24 h and subsequently under hypoxic condition (1% O₂, 5%CO₂, 94% N₂) for 48 h in the incubator (NU4950, NUAIRE company, USA) [29].

**Cell culture**

The parental HCC cell line BEL-7404 and the drug resistant HCC cell line BEL-7404/DOX were obtained from the Department of Physiopathology, Guangxi Medical University (Nanning, Guangxi, China). The doxorubicin-resistant cell line BEL-7404/DOX had been developed by exposure of cells to doxorubicin at gradually

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**Figure 5: The expression of HIF-1α and P-gp proteins.** (A) The expression of HIF-1α and P-gp proteins were measured in BEL-7404/DOX cells by Western blotting analysis. (B) (C) Relative protein levels were quantified by densitometry and showed in the histogram. Values represent means ± SDs of at least three independent experiments performed in triplicate; * p < 0.05 vs. the doxorubicin alone group on the HIF-1α protein; # p > 0.05 vs. the doxorubicin alone group on the P-gp protein; & p < 0.05 vs. the doxorubicin alone group on the P-gp protein. Experiments were repeated three independent times.
increasing doses. BEL-7404 and BEL-7404/DOX cells were maintained in RPMI-1640 medium containing 10% (v/v) FBS and 1% (v/v) PS at 37°C in a normoxic incubator (Forma Scientific, Inc., Marietta, OH). BEL-7404/DOX cells were cultured with doxorubicin (0.1 μg/mL) to maintain drug resistance. For subculturing, cells were dissociated by using 0.25% trypsin.

Cell viability assay

The cytotoxicity of drugs to HCC cells (BEL-7404 and BEL-7404/DOX) was measured by the MTT assay [39]. Cells were seeded in 96-well plates at a density of 5000 cells/well for 48 h. The cells were treated with various concentrations of Y, EGCG, verapamil, and doxorubicin, or doxorubicin in combination with Y, EGCG, and verapamil for 48 h in hypoxic condition. Then MTT (20 μL; 5 mg/mL) was added to each well for an additional 4 h. The medium was removed and 100 μL of DMSO was added to each well to dissolve the formazan crystals that had formed. The optical density at 490nm was measured on a multi-well plate reader (Bio-Rad, Hercules, CA). The percentage of cell survival was calculated using the background-corrected absorbance, as follows: % Cell viability = (OD

All experiments were repeated 3 times. IC_{50} values are drug concentrations at which there is 50% reduction in optical density. The fold of drug resistance was calculated as the IC_{50} of BEL-7404/DOX divided by the IC_{50} of BEL-7404. The degree of reversal of drug resistance (reversal fold or RF) was calculated as the IC_{50} with doxorubicin alone divided by the IC_{50} with doxorubicin in combination with other drugs (Y, EGCG, and verapamil).

Annexin-V-FITC/PI staining assay

The BEL-7404/DOX cells were incubated for 48 h, and then were treated with medium alone, medium with doxorubicin alone, doxorubicin+Y, doxorubicin+EGCG, or doxorubicin+verapamil for 48 h. Subsequently, the cells were subjected to flow cytometry analysis. The BEL-7404/DOX cells were suspended in Annexin V Binding Buffer at a concentration 0.25~1.0 × 10^7 cells/ml. Five μL of FITC and 10 μL of PI were added to 100μL of cell suspension per tube in turn. The cells were mixed in vortex and incubated for 15 minutes at room temperature (25°C) in the dark. Annexin V Binding Buffer (400 μL) was added to each tube. The mixed cell suspensions were analyzed by flow cytometry. The fluorescence of the sample was detected by Epics XL Flow Cytometry (Beckman Coulter) (Ex = 488 nm and Em = 525 nm for Annexin V-FITC; Ex = 488 nm and Em = 620 nm for PI).

RT-PCR analysis

The BEL-7404/DOX cells were incubated in hypoxia (1% O_2) for 48 h, and then were treated with medium alone, medium with doxorubicin alone, doxorubicin+Y, doxorubicin+EGCG, or doxorubicin+verapamil for 48 h. Subsequently, the cells were subjected to RT-PCR analysis. Total mRNA of the BEL-7404/DOX cells was extracted using Trizol reagent according to manufacturer’s instructions, and resuspended in RNase water. The content and purity of total RNA in suspension was measured by ultraviolet spectrophotometer (wave length 260 nm), and the integrity of RNA was checked with the Bioanalyzer 2100. One μg of total RNA was reverse transcribed (RT) to cdNA by using the PrimeScript and RT Primer Mix (including Oligo dt Primer and Random 6 mers). For PCR amplification of cdNA, 2 μL of cdNA liquid was added to 23 μL of PCR reaction media (including SYBR Premix Ex Taq, PCR Forward/Reverse Primer, and double distilled water). PCRs were carried out in the conditions according to the instructions: initial denaturation (95°C for 30 s) followed by 40 cycles of denaturation (95°C for 5s), and hybridization-extension (60°C for 31s) using Applied Biosystems7300 Real Time PCR System. The gene names (including target genes hif-1α and MDRI and housekeeping gene ACTB), access number, primer sequence, and amplifying size are listed in Table 4. In the results, transcription levels of target genes were normalized to those of ACTB to compensate for difference in efficiency of reverse transcription and input RNA amounts; they were expressed relative to the control. The relative quantitation of target gene expression was calculated by the comparative ΔΔCT method.

Western blot analysis

The BEL-7404/DOX cells were incubated in hypoxia (1% O_2) for 48 h, and then were treated with medium alone, medium with doxorubicin alone, doxorubicin+Y, doxorubicin+EGCG, or doxorubicin+verapamil for 48 h. Subsequently, the cells were subjected to Western blot analysis. Cells with different treatments were lysed in an ice-cold buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin, 1× protease inhibitor cocktail, and 1×phosphatase inhibitor cocktail at 4°C for 30 min. Cell lysates were centrifuged at 4°C with 13,000 rpm/min for 10 min. Supernatant of cell lysates was transferred to the Eppendorf tube and measured for protein concentrations.

Twenty-five micrograms of protein liquid from each sample were placed in electrophoresis tanks in a 10~12% SDS polyacrylamide gel. The cell lysates were separated on the SDS polyacrylamide gel and then transferred electrophoretically onto a nitrocellulose membrane, which was pre-hybridized in methyl alcohol. The membrane was submerged in 5% skim milk for 1 h, and then transferred to a solution containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween-20 (TBST buffer) and a primary antibody and incubated overnight at 4°C. After washing with the TBST buffer, the membrane
was submerged in TBST buffer containing a Dy Light 680 secondary antibody in the dark at room temperature for 2 h. The membrane was washed with TBST buffer and then exposed to Infrared Imaging System to visualize the bands.

**Statistical analysis**

The results of each experiment were represented as the mean ± standard deviation (SD) and analyzed in SPSS 20.0 software. One-Way ANOVA method was used in multiple comparison tests. A difference was considered significant when \( p \) value was less than 0.05.

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**CONFLICTS OF INTEREST**

There is no conflicts of interest.

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| Gene   | Genebank access number | Primer sequence (5’–3’)                              | Amplicon size (bp) |
|--------|------------------------|------------------------------------------------------|--------------------|
| hif-1α | NM_001243084.1 [42]    | Forward- GACACAGAAGCAAGAAACCCCA                     | 241                |
|        |                        | Reverse- CATCAGTGGTGGCAGTGTA                         |                    |
| MDR1   | NM_000927.4 [43]       | Forward- CTCTTTGCCACAGGAAGCCT                        | 187                |
|        |                        | Reverse- CATTGGCTCCCTCCTCAAGA                        |                    |
| ACTB   | NM_001101.3 [44]       | Forward- GGCAATCCTACCCCTGAAGTA                      | 102                |
|        |                        | Reverse- GCACACGCAGCTCATGTAG                         |                    |
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