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The Involvement of ROS Generation in Difenoconazole-Induced Hepatocellular Toxicity

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Abstract

Difenoconazole is one of the triazole compounds, and is widely used as environmental fungicides. Recently, it is suspected that long-term exposure to difenoconazole through drinking water and food may induce health effects including hepatotoxicity and tumorigenesis. However, there is a little information regarding the molecular mechanism of difenoconazole-induced cytotoxicity and/or human health risk. The present study was aimed to investigate the toxic effects of difenoconazole on human hepatocellular carcinoma HepG2 cells in terms of cell viability, generation of reactive oxygen species (ROS) and cellular apoptosis. Difenoconazole significantly activated the apoptosis related proteins namely caspase-8 and -9 as well as cleavage of poly-ADP ribose polymerase (PARP) in a dose-dependent manner whereas the IC_{50} value for difenoconazole was calculated to be 70μM. Correspondingly, intracellular generation of ROS was also markedly increased after exposure to difenoconazole and was observed to occur specifically in mitochondria and endoplasmic reticulum (ER), suggesting mitochondria and ER being the target organelles for difenoconazole-induced cytotoxicity. Moreover, phosphorylation of protein kinase-like endoplasmic reticulum kinase (PERK), followed by initiation of ATF4, phosphorylation of eif2α and activation of Chop were observed to be up-regulated by difenoconazole, indicating that difenoconazole is capable of inducing ER-stress in HepG2 cells. More interestingly, either activation of caspase-8, -9 and cleavage of PARP or induction of ER-stress can be attenuated by pretreatment with antioxidants either N-acetylcysteine (NAC) or lipoic acid (LA), indicating that generation of ROS may result in induction of apoptosis and cell death probably through ROS-mediated mitochondria and ER-stress dependent signaling pathway.

Keywords: carcinogen, cytotoxicity, difenoconazole, ER-stress, liver cancer, reactive oxygen species

Abbreviations: ER, endoplasmic reticulum; NAC, N-acetylcysteine; LA, lipoic acid; PERK, protein kinase-like endoplasmic reticulum kinase; PARP, poly-ADP ribose polymerase; ROS, reactive oxygen species

Introduction

Triazoles are commonly used as environmental fungicides for grain, vegetable, fruit, and flower production worldwide.[1] Likewise, it has also been applied for treatment of human mycoses including vaginal mycoses in pregnant women and thrush in infants as pharmaceutical fungicides (Hester et al., 2012).[2] However, the use of triazole antifungal agents has recently been considered to be an important public health problem, because a number of triazole fungicides exhibited a wide range of toxicological properties in mammalian and aquatic systems inducing hepatic, thyroid and developmental toxicities.[3,4] However, there is a little information regarding the toxic effect of triazoles-induced cytotoxity and/or human health risk. Thereby, more mechanistic studies are needed to address the exact toxic mechanism of triazoles *in vivo* or *in vitro*.

US Environmental of protection agency (EPA) has reported that triazoles are able to increase the incidence of the hepatocellular adenomas in mice and rats after feeding high levels of triadimefon for two years.[5] In addition, it has also been reported that propiconazole can increase the incidences of benign and malignant liver tumors in male CD-1 mice at the highest feeding level (e.g., 2500 ppm),[6] but unfortunately, no tumors were observed to be formed in the rats at any feed level even after long term,[7] suggesting that the carcinogenic effects associated with fungicides probably vary depending on the animal species. In addition, very recently Currie has indicated that phenobarbital (PB) and propiconazole (PPZ) can cause malignant liver tumors in the mice after long term feeding with high levels.[8]

In fact, conazoles are known to inhibit a specific cytochrome P450 (CYP) enzyme, CYP51 (i.e., lanosterol-14a-demethylase), which is involve in the major step of ergosterol biosynthesis required for the synthesis of the fungal cell membrane.[9] Several reports have indicated that triazoles including fenbuconazole, propiconazole, fluconazole, triadimefon, and myclobutanil were found to be capable of inducing specific CYP isoforms in mouse and rat liver.[10-13] In addition, two fungicides; tebuconazole and epoxiconazole have also exhibited to influence activity of estrogen-synthesizing enzyme, implying that the chronic exposure of triazoles may induce various diseases in mammals.

Difenoconazole (cis-trans-3-chloro-4-(4- methyl-2-(1H-1,2,4-triazol-yl methyl) -1,3-dioxolan-2-yl) phenyl 4- chlorophenyl ether) , a typical fungicide, has been found in tea leaves, in made green tea as well as tea infusions. It has been reported that with

the increase in the brewing time there occurs increases in the transfer of difenoconazole from the made tea into the brew.[14] Moreover, since it is being used extensively in crops, difenoconazole has also been found in rice, paddy soil and paddy water, making difenoconazole as an emerging public health issue. Hinfray has indicated that difenoconazole is able to inhibit brain and ovarian aromatase activities in fish,[15] indicating that the difenoconazole might also have the potential for toxicity in mammals. Similarly, on further studying the toxic effects of difenoconazole on embryonic development of zebrafish, it was found that exposure to difenoconazole could induce a large suite of negative influences such as hatching regression, heart rate decrease, growth inhibition and developmental abnormalities.[16] Therefore, the difenoconazole is considered to be an important toxic compound, and the molecular mechanisms of their toxic effects should be clearly identified.

Liver is well known as a prime site for the conversion of xenobiotics to their relevant metabolites by CYP450 enzyme for their excretion into bile or blood stream. Likewise, liver is also recognized to be one of the target organs affected by the toxic effects of pesticides.[17] It has been suggested that generation of ROS is associated with DNA damage and deletion mutations,[18] thus, the generation of ROS is suggested to play an important role in induction of carcinogenesis.[19] In addition, it has found that oxidative stress related genes (Akr1b7, Hspa1 and Gstα) were strikingly up regulated after exposure to propiconazole, implying that some of the triazoles are probably able to induce oxidative stress in animals.[4]

In the present study, in order to understand the mechanism underlying the toxic effect of difenoconazole, we used human hepatocellular carcinoma HepG2 cells to determine the toxic effects of difenoconazole on cell viability, generation of reactive oxygen species, translocation of pro- and anti-apoptotic proteins such as Bax, Bcl-2 and Cyt c in mitochondria and cytoplasm, induction of ER-stress and apoptosis. Here, we have provided new evidence that difenoconazole-induced cell death predominantly occurs through mitochondria and ER-stress signaling pathways through increase generation of ROS, indicating that difenoconazole might have potential toxic effects on the two organelles (i.e., mitochondria and ER).

Material and Methods

Reagents

All reagents were of analytical grade. Milli-Q water (Millipore) was used throughout the experiment. Trizma® HCl and Trizma® Base were purchased from Sigma (St. Louis, MO, USA). Difenonconazole was purchased from Labor Dr. Ehrebstorfer-Schafers.

Cell culture

Human hepatoma HepG2 cells were purchased from Chinese Academy of Sciences in Shanghai. Cells were cultured in logarithmic growth phase using RPMI-DMEM medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, USA), 100U/mL penicillin, and 100μg/mL streptomycin, at 37°C in 5% CO2 atmosphere. After twenty-four hours of seeding, the cultures were washed twice with PBS and fresh medium was added. The cells were then treated with indicated concentration of difenoconazole for 24h.

MTT assay for cell viability

Human hepatoma HepG2 cells were seeded at a density of 2×104 cells/180μL/well in 96-well microtiter plates (Promega Corporation). Twenty-four hours post-seeding, the cultures were washed twice with PBS and then exposed to different concentration of difenoconazole (e.g., 0, 10, 25, 50, 75, 100μM) for 24h. Then, 20 μL of an MTT solution was added to each well (at the final concentration of 0.5mg/mL), and the plates were incubated for an additional 3h at 37ºC. Afterward, HepG2 cell cultures were washed with PBS, and 150μL of DMSO was added to each well. Cell viability was measured as absorbance at 570 nm with a microplate reader and the results were expressed as a percentage of the control level.

Measurement of mitochondrial membrane potential (ΔΨm) by flow cytomerty

HepG2 cells were treated with different concentration of (e.g., 0, 25, 50, 100μM) difenoconazole for 6h. After incubation, cells were washed with PBS, loaded with 10 mg/mL JC-1 dye and incubated 37ºC in the dark for 15 min. After dying, the cells were analyzed by flow cytometry.

Western blot analysis

Difenoconazole treated-HepG2 cells were washed twice with cold D-hanks solution, followed by the cells were lysed in RIPA lysis buffer (containing 50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM PMSF,

and a complete mini protease inhibitor tablet), and incubated on ice for 30 min, centrifuged at 13,000×g at 4ºC for 30 min to obtain the supernatant. Protein concentrations were determined by Bio-Rad microprotein assay using bovine serum albumin as the standard. Twenty-five microgram of each protein sample was resolved by 10 or 12% SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad, Mississauga, ON). The membranes were blocked for 1 h at room temperature in PBS containing 5% skim milk plus 0.1% Tween-20 (PBST) and incubated overnight at 4ºC with different primary antibodies, followed by incubation with HRP-linked secondary antibodies for 1h at room temperature and then the proteins were visualized by enhanced chemiluminescence (ECL).

Antibodies

Primary antibodies rabbit anti-Poly (ADP-ribose) polymerase (PARP) polyclonal antibody, Cyt c, and PERK were purchased from Santa Cruz Biotechnology (CA, USA). Primary antibodies CHOP, ATF4, Bax, BCL-2, β-actin, anti-cleaved-8 antibody, caspase-8, anti-cleaved caspase-9, caspase-9, P-eiF2α, GAPDH, Cox IV were purchased from Cell Signaling Technology (Danvers, MA).

Measurement of intracellular ROS by flow cytometry

The oxidation-sensitive fluorescent probe (DCFH-DA) was used to detect the intracellular ROS level, as described in our previous work.[20] Briefly, HepG2 cells were treated with Difenoconazole for 12h. After incubation, cells were washed with PBS and then incubated with 10μM/L DCFH-DA at 37 ºC for 20 min. Fluorescence was detected by flow cytometry.

Fluorescence microscopy

HepG2 cells were treated with different concentration of difenoconazole for 24h, Cells nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, blue) for 5 min at 4 °C. After incubation, cells were washed with PBS for two times and then analyzed by fluorescence microsocopy.

Statistical analysis

Each viability value represents the mean±S.D. from four determinations, and IC50 values were calculated from the log-log plot between the percentages of viable cells. Subsequently, each experiment was performed at least three times. Statistical analysis of data was carried out using a one-way ANOVA followed by Holm-Sidak pairwise

multiple comparison test (Sigmaplot, Systat Software Inc), and a probability value of less than 0.05 (*$p<0.05$) was accepted as a significant difference.

Results

Effect of difenoconazole on cell viability of HepG2 Cells

In the current study, difenoconazole was dissolved in the 0.1% of DMSO to be used in subsequent experiments. The chemical structural of difenoconazole is shown in Figure 1A. To assess the toxic effects of difenoconazole on cell survival, HepG2 cells were exposed to various concentration of difenoconazole for 24h, and the cell viability was determined by MTT assay (Fig.1B). The cells survival was significantly reduced with the increase in the exposure doses, especially the cell viability was reduced to approximately 80% of control at 50μM, and the IC_{50} value for difenoconazole was calculated to be 70μM. Additionally, apoptoticlike morphological changes in the nuclei of HepG2 cells were clearly observed by DAPI staining after exposure to high concentration of difenoconazole (Fig.1C).

Effect of difenoconazole on apoptotic related proteins in HepG2 cells

To gain further insight into the molecular mechanism of difenoconazole in *HepG2* cells, we further determined the apoptotic related proteins such as cleaved poly (ADP-ribose) polymerase (PARP), caspase-8 and 9 in HepG2 after exposure to difenoconazole for 24h in a dose dependent manner (i.e., 0,25,50 and 100μM) (Figure 2A). The cleaved PARP was significantly observed at 50μM, while caspase-8 and 9 were found to be completely cleaved at 100μM, suggesting that the cell death probably occurred through apoptotic pathway. Moreover, effects of difenoconazole on cells were further determined in a time dependent manner at subtoxic dose of 50μM, where the cleavage of PARP, caspase-8 and 9 was found as early as 12 h which later increased with the increase in exposure time (Fig.2B).

Changes in Cyt c, Bcl-2 and Bax in mitochondria and cytoplasmic fractions in HepG2 cells by difenoconazole

Additionally, the pro- and anti-apoptotic proteins including bax, bcl-2 or cyt c in cytoplasm and mitochondria of HepG2 cells were also examined, as shown in Figure3. Difenoconazole remarkably induced the translocation of propoptotic protein bax from

cytoplasm to mitochondria, while anti-apoptotic protein bcl-2 was decreased in both fractions at 50~100μM (Fig.3A and B). Meanwhile, cyt c was reduced in mitochondria and increased in cytoplasm (Fig.3A, B). Moreover, the same results were also observed in cells exposed to 50μM of difenoconazole in a time-dependent manner (Fig.3C, D). Correspondingly, loss of mitochondrial membrane potential (ΔΨ) was also induced by difenoconazole at different concentrations, i.e., 25 (27.1%), 50 (34.5) and 100 μM (97.4%) as compared with control (18.3%), as shown in Figure3E.

ROS generation and localization in HepG2 cells by difenoconazole

In order to better understand the mechanism underlying difenoconazole-induced apoptosis in HepG2 cells, we assayed the generation of intracellular ROS by exposure to difenoconazole at 50 μM for 6h (Figure 4). Intracellular ROS was remarkably increased after exposure to difenoconazole, while it was reduced to the control level by pretreatment with N-acetylcysteine (NAC) before exposure to difenoconazole (Fig.4A).

We were further interested in investigating the subcellular localization of ROS in HepG2 cells. Thus, Mito-, Golgi and ER-tracker were used to establish the localization of ROS in organelles (i.e., mitochondria, Golgi apparatus and endoplasmic reticulum) using confocal laser scanning microscopy. Interestingly, generation of ROS was specifically found to be localized in both mitochondria and ER (but not in Golgi apparatus, data not shown), as indicated by yellow fluorescence (Fig.4B, C), implying that difenoconazole has potential toxic effects on both mitochondria and ER organelles.

Effect of antioxidants and caspase inhibitor on induction of ROS by exposure to difenoconazole

Based on the above results, we found that difenoconazole-induced apoptosis is probably associated with ROS. To confirm the involvement of ROS in apoptosis, we determined the changes in the cleaved PARP by exposing HepG2 cells to difenoconazole in the presences of antioxidants NAC and lipoic acid (LA), as shown

in Figure 5. Both NAC and LA showed to significantly prevent the induction of apoptosis (Fig.5 A, B), suggesting that ROS is probably the main cause for the induction of apoptosis.

Moreover, to find out that whether the cellular apoptosis occurred through caspase-dependent pathway, HepG2 cells were pretreated with the inhibitor of caspase Z-VAD-FMK and were then expose to difenoconazole (50 μM) for 24h (Fig.5C). As anticipated, the induction of apoptosis was markedly prevented by Z-VAD-FMK treatment, indicating that apoptosis was mainly induced through caspase-dependent pathway.

Induction of ER-stress in HepG2 cells by difenoconazole

Apoptosis can be induced by different signaling pathways (e.g., mitochondria or ER dependent pathways). Thus, we further focused on the effect of difenoconazole on the induction of ER stress. The phosphorylation of PERK was found to be induced as early as 1h which further increased with the increase in the exposure time up to 9h (Figure 6A). Correspondingly, the downstream proteins such as ATF4, phosphorylated eiF2α and Chop were also observed to be significantly activated, indicating that difenoconazole is able to induce the ER-stress in HepG2 cells. However, it is unclear whether the ER-stress resulted in generation of ROS or ROS induced the ER stress in HepG2 cells. Thus, antioxidants; NAC and LA were used to examine the relationship between the ER-stress and generation of ROS. Interestingly, both NAC and LA could inhibit the induction of ER-stress as well as apoptosis, suggesting the generation of ROS results in inducing cell death after exposure to difenoconazole.

Discussion

Triazole fungicides belonging to the group called conazoles, are widely used as environmental fungicides,[21] whereas high levels of these fungicides have been found in ground water, soil or cabbage, etc.[22,23] In addition, triazole fungicides demonstrates a wide range of toxicological properties in mammalian systems,[3] suggesting that the molecular mechanism underlying the toxic effects of triazole fungicides needs to be clearly identified.

Difenoconazole is one of triazole fungicides commonly found in tea leaves and food (e.g., Chilli, Jam and Cabbage).[23-25] However, there is a little information available regarding the toxic effects of difenoconazole on human health. Very recently, Mu has reported that difenoconazole is able to induce cytotoxicity in zebrafish, they found that difenoconazole could induces hatching inhibition, abnormal spontaneous movement, slow heart rate, growth regression and morphological deformities in Zebrafish after exposure to difenoconazole, implying that the difenoconazole probably may also have adverse effects on human health.[16]

In the current study, effects of difenoconazole have been studied on human hepatocellular carcinoma HepG2 cells, and our results clearly indicated that difenoconazole could strongly affect cell survival in a dose- and time-dependent manner (Fig.1). In fact, *in vivo* studies have indicated that long term exposure of triadimefon (one of triazole) to mice is able to increase the incidence of hepatocellular adenomas in CF1/W74 mice, but this chemical compound did not show any genotoxicity *in vitro*.[26] Moreover, it has been suggested that induction of liver hypertrophy in mice might be associated with inhibition of CYP activity by triadimefon. A few studies have reported that propiconazole and fluconazole could induce CYP2B and CYP3A activities in mice and rats,[27,28] while tioconazole is able to inhibit activities of CYP2 (A,C,D) and 3A, indicating that different conazole have varying effects on CYP enzymes.[29] In this study, induction of CYP3A4 expression and activity in HepG2 cells has also been evaluated after exposure to difenoconazole. Unexpectedly, no appreciable changes were observed in the protein expression in HepG2 cells following exposure to difenoconazole, while CYP3A4 activity was

found to be significantly inhibited at low doses of difenoconazole (data not shown). Besides, it seems that the inhibition of CYP3A4 activity dose not correlate with cytotoxity of difenoconazole in the current study.

Poly (ADP-ribose) polymerase-1 (PARP-1) normally functions in DNA repair, but extensive PARP-1 activation is a major cause of cell death.[30] In this study, cleaved PARP-1 was significantly increased in cells after exposure to difenoconazole, followed by activation of caspase-8 and 9 (Fig.2). Correspondingly, proapoptotic protein bax was translocated from cytoplasm to mitochondria, while cyt c was reduced in mitochondria and was increased in cytoplasm (Fig.3), suggesting that difenoconazole could induce apoptosis. Likewise, induction of apoptosis can be markedly prevented by pre-treatment of Z-VAD-FMK (inhibitor of caspase) (Fig.5C), signifying that the cell death by difenoconazole is suggested to occur through caspase-dependent pathway.

On the other hand, oxidative stress has been implicated to play an important role, at least in part of pathogenesis of many disease conditions and toxicities in animals.[31] Ward has reported that mice exposed to propiconazole may induce high levels of CYPs, these changes probably result in the induction of oxidative stress.[4] Our results have clearly shown that difenoconazole markedly increases the amount of intracellular ROS which was found to be generated specifically in endoplasmic reticulum and mitochondria (Fig.4), indicating that difenoconazole may have potential toxic effects on these two organelles. Moreover, when cells were pretreated with antioxidants; N-acetylcysteine or lipoic acid, the generation of ROS, induction of apoptosis and cleaved PARP were significantly attenuated after exposure to difenoconazole (Fig.5). Thus, we assumed that difenoconazole may directly inhibit the activity of the mitochondrial electron transport chain (ETC), which resulted in increasing ROS production in mitochondria. However, further study is needed to reveal the effect of difenoconazole on inhibition of ETC in intact mitochondria.

In addition, difenoconazole is able to induce ER-stress in HepG2 cells, where the phosphorylation of PERK along with activation of downstream proteins; ATF4, phosphorylated eif2α and Chop significantly occurred at early time, however, were

found to be attenuated by pretreatment with antioxidants (e.g., NAC and LA), indicating the generation of ROS as the main cause of cell death by difenoconazole.

Based on the present study, it can be concluded that difenoconazole is capable of inducing cell toxicity in HepG2 cells, predominately by generation of ROS, which then induced mitochondrial dysfunction as well as ER-stress finally resulting in cell death. Although here the difenoconazole concentration that induced cellular toxicity seems to be much higher than that found in the environment or food, it is important to take into consideration that short exposures to difenoconazole for long term can contribute in inducing related adverse effects, however further studies are required to unravel the toxic effects of difenoconazole on animals *in vivo*.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures Legends

Figure1. Effect of difenoconazole on human hepatocellular carcinoma HepG2 cells. (**A**) Chemical structural of difenoconazole in environment. HepG2 cells were exposed to indicate concentrations of difenoconazole for 24h (**B**). DAPI staining was performed to determine the pyknotic nuclei in HepG2 cells following exposure to difenoconazole (**C**), and arrowhead indicates the nuclei apoptoticlike morphological changes.

Figure2. Determination of apoptotic related proteins in HepG2 cells after exposure to difenoconazole. Cells were exposed to difenoconazole in a dose-dependent manner (0~100 μM) for 24h (**A**) or at 50μM of difenoconazole in a time-dependent manner (0~48h) (**B**). The apoptotic related proteins such as poly (ADP-ribose) polymerase (PARP), caspase-8 and -9 or corresponding cleaved proteins were determined by immunoblotting using specific antibodies. β-actin was used as a loading control.

Figure3. Changes in cytc, bcl-2 and bax in mitochondria or cytoplasmic fractions in HepG2 cells. Cells were exposed to difenoconazole in a dose-dependent manner (0~100 μM) for 24h (**A,B**) or at 50μM of difenoconazole in a time-dependent manner (0~48h) (**C,D**) for 24h. Anti-apoptotic proteins Bcl-2, pro-apoptotic proteins Bax and Cyt c in mitochondrial (**A,C**) or cytoplasm fractions (**B,D**) were determined by immunoblotting. β-actin and COX IV were used as loading controls. The mitochondrial membrane potential ($\Delta\Psi_m$) was determined in HepG2 cells by using the fluorescent dye JC-1 at 6h after exposure to difenoconazole (**E**).

Figure4. Effect of antioxidants and caspase inhibitor on induction of apoptosis by difenoconazole. Cells were exposed to difenoconazole at 50μM for 6h to determine generation of ROS by flow cytometry (**A**). HepG2 cells were seeded on a 6-well culture plates with cover glasses, and then cultured for 24h. Cells were pre-treated with 2μM CM-H2DCFDA (green) and ER-tracker Red (red) (**B**) or Mito-tracker (red) (**C**) for 30 min. After washing with PBS, cells were exposed to mentioned concentration as indicate above for 6h. Following exposure, cells were fixed with 10% formalin, and then ROS generation was determined using a confocal laser scanning

microscope.

Figure5. Induction of apoptosis in HepG2 cells after exposure to difenoconazole in presence of antioxidants or caspase inhibitor. Cells were exposed to 50μM for difenoconazole alone or with N-Acetylcysteine (NAC) (**A**), Lipoic acid (LA) (**B**) or inhibitor of caspase (Z-VAD-FMK) (**C**) for 24h. Changes in cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-9 were determined by immunoblotting using specific antibodies. β-actin was used as a loading control.

Figure6. Induction of ER-stress in HepG2 cells after exposure to difenoconazole. Cells were exposed to difenoconazole at 50μM for the indicated time points (i.e., 0,1,3,6,9,12 and 24h) to determine the changes in p-Perk, ATF4, p-eif2a and Chop (**A**) or in the presence of NAC (**B**) and LA (**C**) to determine the ATF4 and Chop (**A**) by immunoblotting. GAPDH was used as a loading control.

Figure1

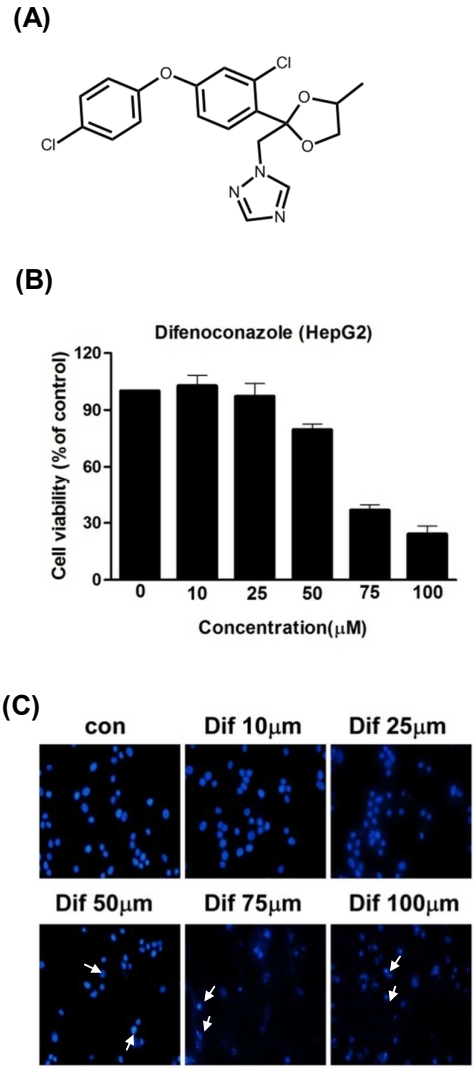

Figure 2

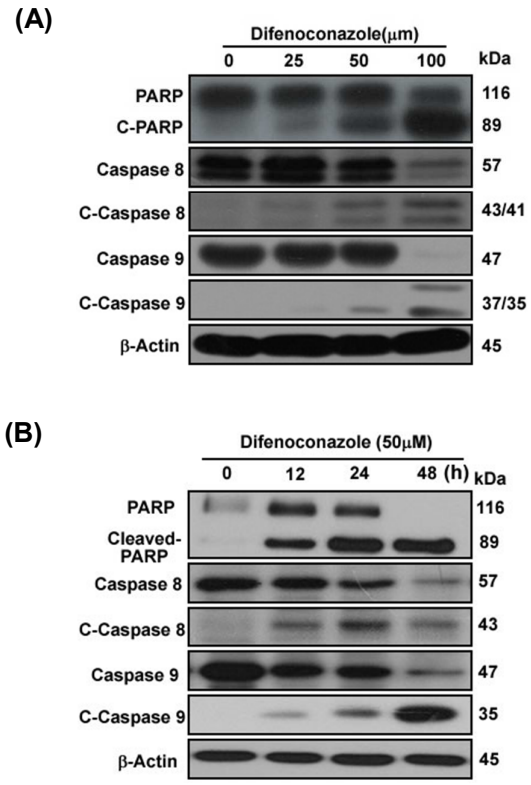

Figure 3

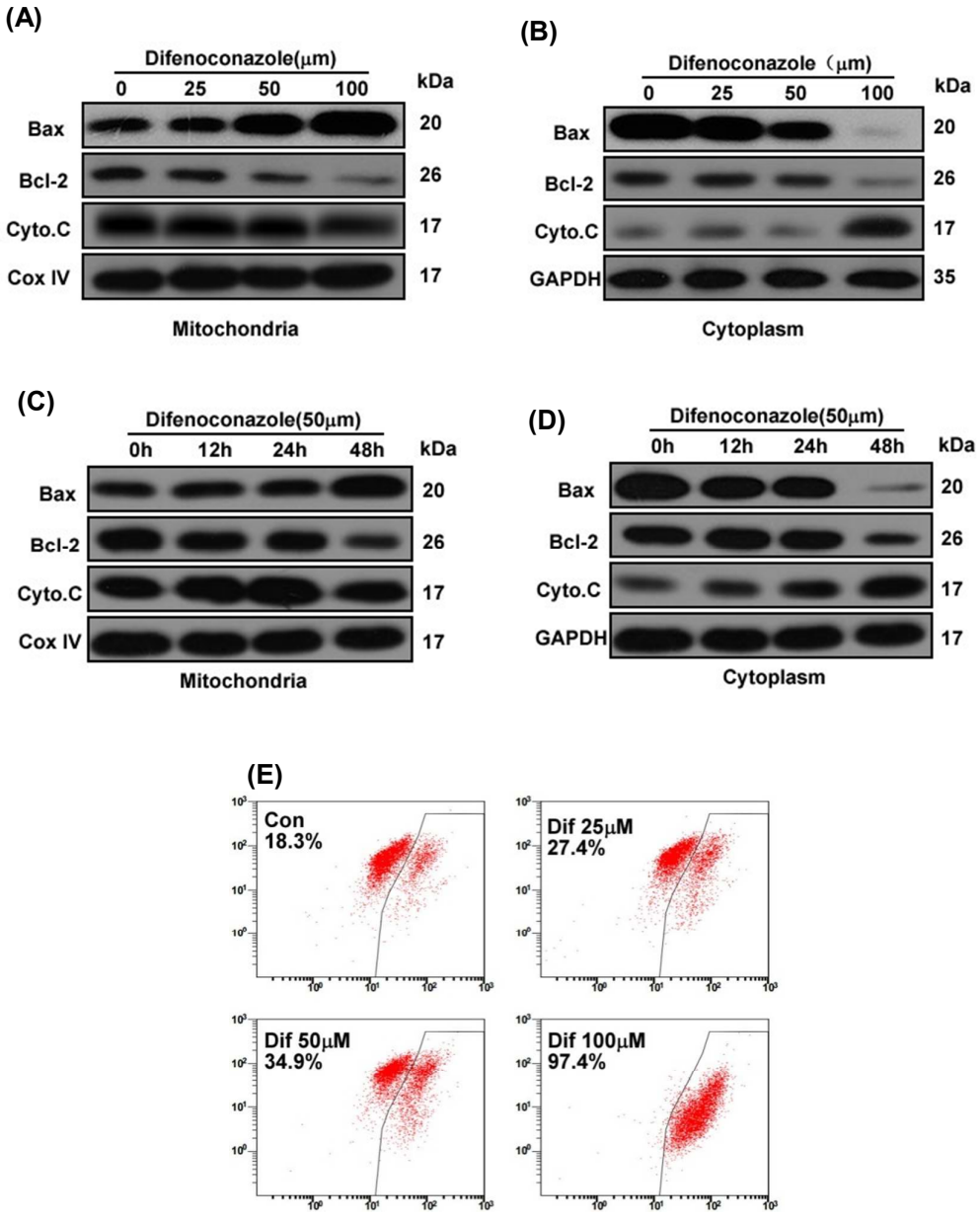

Figure 4

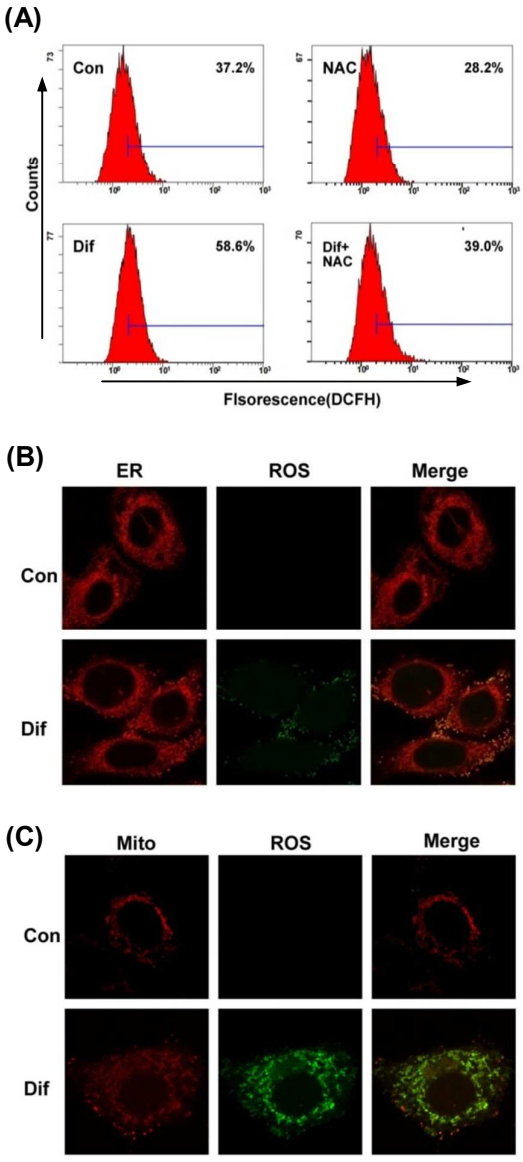

Figure 5

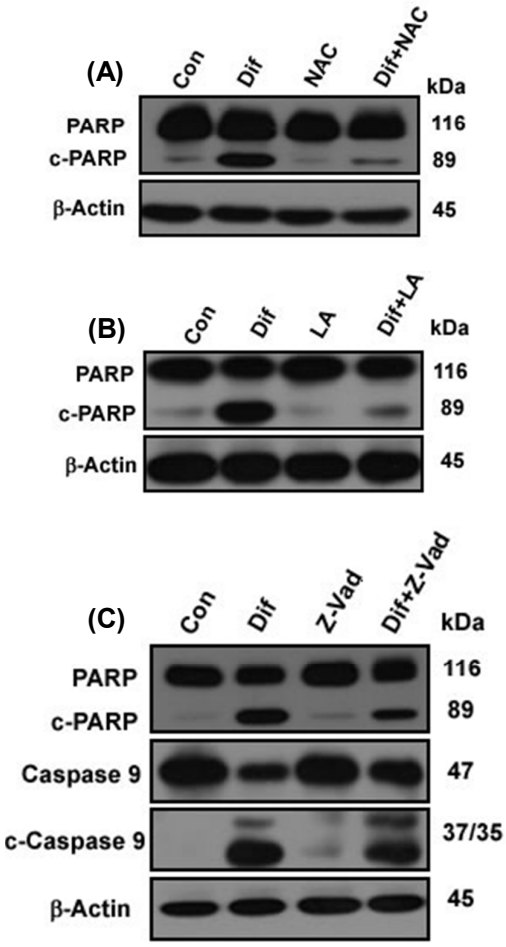

Figure 6