Research Article

YQ36: A Novel Bisindolylmaleimide Analogue Induces KB/VCR Cell Death

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Overexpression of multidrug resistance proteins P-glycoprotein (P-gp, MDR1) causes resistance of the tumor cells against a variety of chemotherapeutic agents. 3-(1-methyl-1H-indol-3-yl)-1-phenyl-4-(1-(3-(piperidin-1-yl)propyl)-1H-pyrazolo[3,4-b]pyridine-3-yl)-1H-pyrole-2,5-dione (YQ36) is a novel analogue of bisindolylmaleimide, which has been reported to overcome multidrug resistance. Here, we dedicated to investigate the anticancer activity of YQ36 on KB/VCR cells. The results revealed that YQ36 exhibited great antiproliferative activity on three parental cell lines and MDR1 overexpressed cell lines. Moreover, the hypersensitivity of YQ36 was confirmed on the base of great apoptosis induction and unaltered intracellular drug accumulation in KB/VCR cells. Further results suggested that YQ36 could not be considered as a substrate of P-gp, which contributed to its successfully escaping from the efflux mediated by P-gp. Interestingly, we observed that YQ36 could accumulate in nucleus and induce DNA damage. YQ36 could also induce the activation of caspase-3, imposing effects on the mitochondrial function. Collectively, our data demonstrated that YQ36 exhibited potent activities against MDR cells, inducing DNA damage and triggering subsequent apoptosis via mitochondrial pathway.

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1. Introduction

A major obstacle in cancer treatment is the resistance to chemotherapeutic drugs. Frequently, tumor cells exhibit resistance not only against a single class of drugs but also against a variety of chemotherapeutics, such as vincristine, doxorubicin, daunorubicin, taxol, and VP-16 [1, 2]. This phenomenon, known as multidrug resistance (MDR), is generally caused by three major mechanisms in cells [3]: first, decreased uptake of water-soluble drugs such as folate antagonists, nucleoside analogues, and cisplatin, which require transporters to enter cells; second, various changes in cells affect the capacity of cytotoxic drugs to kill cells, including alterations in cell cycle, increased repair of DNA damage, reduced apoptosis and altered metabolism of drugs; and third, increased energy-dependent efflux of hydrophobic drugs that can easily enter the cells by diffusion through the plasma membrane.

Of these mechanisms, one that is the most commonly encountered in laboratory is the increased efflux of a broad class of hydrophobic cytotoxic drugs that is mediated by one of a family of energy-dependent transporters, known as ATP-binding cassette (ABC) transporters. The best-characterized ABC transporter is the P-glycoprotein (P-gp), which is encoded by the multidrug resistance gene 1 (MDR1 or ABCB1) [3].

It is reported that reversible phosphorylation is a possible mechanism for regulating the transport and chloride channel regulation functions of P-gp whereas protein kinase C (PKC) is a good candidate for inducing such phosphorylation [4]. Therefore, inhibition of PKC is regarded as an approach for overcoming MDR in cancer chemotherapy. Bisindolylmaleimide (BIM) was reported to exhibit remarkable PKC inhibition [5]. However, little evidence showed that BIM reversed MDR in cancer cells through inhibiting PKC activity. In addition, most BIM analogues reversed MDR via interacting with P-gp directly. Importantly, these results could not support the concept of a major contribution of PKC to a P-gp-associated MDR [6, 7].
In human cells, both the normal metabolic activities and the environmental factors including UV light, γ-radiation, and chemotherapeutic agents like mitomycin C could cause DNA damage [8]. There are many types of damage to DNA due to exogenous agents or endogenous cellular processes, mainly including base loss, base modification, replication errors, inter-strand crosslinks, DNA-protein crosslinks, and strand breaks. The p53 tumor suppressor protein plays a major role in cellular response to DNA damage and other genomic aberrations. Activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis. Extensive studies reveal that p53 can be phosphorylated by ATM, ATR, and DNA-PK and the phosphorylation of p53 promotes both the accumulation of damaged DNA and the activation in response to DNA damage [9].

Here, the newly designed and synthesized BIM analogue, 2,5-dione (YQ36, Figure 1), exhibited obvious anti-MDR effect on a panel of cell lines with or without MDR1 expression [10].

Figure 1: The structure of YQ36.

2. Materials and Methods

2.1. Drugs and Chemicals. YQ36 and BIM, a generous gift from Professor Hu (ZJU-ENS Joint Laboratory of Medicinal Chemistry, School of Pharmaceutical Sciences, Zhejiang University), were synthesized as a recent published article (“Synthesis and evaluation of novel 7-azaindazolyl-indolylmaleimide derivatives as antitumor agents and protein kinase C inhibitors”: Q. Ye et al. Bioorg. Med. Chem. 2009. in press). YQ36 and BIM were dissolved in DMSO (10.0 mM stock solution) and stored at −20°C. Doxorubicin (DOX) was purchased from Sigma (St Louis, MO, USA). Stock solution of DOX (10mM) was prepared in DMSO and stored at −20°C. The primary antibodies to caspase-9, caspase-3, Bcl-2, Bax, p53, p-p53, Apaf-1, Akt, p-Akt, p-Jnk, HSP60 and β-Actin, and HRP-labeled secondary antibody, antimus, and antirabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Appropriate antibodies to cleaved caspase-3, Smac and XIAP from Cell Signaling Technology (Beverly, MA) and P-gp from Merck (Darmstadt, Germany) were also used. ECL, a western blot detection reagent, was purchased from Pierce Biotechnology (Rockford, IL). The caspase inhibitor BOC-D-FMK and Z-DEVD-FMK were purchased from Merck (Darmstadt, Germany) and were dissolved in DMSO (100.0 mM and 40.0 mM stock solution resp.).

2.2. Fluorescence Microscope. Cells were cultured in 6-well plates. After treated with YQ36 (40 μM) for 1 hour, samples were examined in a fluorescence microscope and photographed using a Digital Color Camera DFC 300FX (Leica, Germany).

2.3. Cell Lines. KB, KB/VCR, K562, and K562/ADR cells were maintained in RPMI 1640 (Gibco, 2 g/l glucose) and MCF-7, and MCF-7/DOR were maintained in modified Eagle’s medium (Gibco, 4.5 g/L glucose); both media were supplemented with 10% heat-inactivated newborn calf serum (NCS, Gibco) plus 100 U/mL penicillin. All of the cell lines were purchased from the Institute of Cell Biology (Shanghai, PRC) and grown at 37°C in a 5% CO2 atmosphere.

2.4. Antiproliferation Assay. Cells were cultured in 96-well plates and the Antiproliferation activity of YQ36 and doxorubicin on KB and KB/VCR cells were detected using the MTT assay. After treatment in 96-well plates, MTT solution (5.0 mg/mL in RPMI-1640, Sigma, St. Louis, MO) was added (20.0 μL/well), and the plates were incubated for an additional 4 hours at 37°C. The purple formazan crystals were dissolved in 100.0 μL DMSO. After 5 minutes, the plates were read on a Multiskan Spectrum (Thermo Electron Corporation, Marietta, Ohio) at 570 nm. Assays were performed in triplicate on three independent experiments. The concentration of drug exhibited 50% inhibition of cells (IC50) was calculated using the software of Dose-Effect Analysis with Microcomputers.

2.5. Real-Time RT-PCR. Real-Time RT-PCR was executed as in a previous report [10]. Briefly, total RNA was extracted from sample cells with Trizol. The RNA was precipitated by isopropyl alcohol and rinsed with 70% ethanol. Single-strand cDNA was prepared from the purified...
Table 1: The IC50 values of YQ36 and DOX on three panels of chemosensitive tumor cell line and their chemoresistance subline. The fold indicated the index of IC50 on chemoresistance subline versus chemosensitive cell line.

| Cell lines     | YQ36       | IC50 (μM) YQ36 | Fold | IC50 (μM) Doxorubicin | Fold |
|----------------|------------|----------------|------|-----------------------|------|
| KB             | 5.80 ± 0.39| 4.33 ± 0.16    | 0.82 | 28.69 ± 1.87          | 6.60 |
| KB/VCR         | 4.78 ± 0.66| 29.76 ± 1.48    | 0.50 | 133.48 ± 13.95        | 4.49 |
| MCF-7          | 15.68 ± 1.41|                |      |                       |      |
| MCF-7/DOR      | 7.90 ± 0.73| 3.06 ± 0.04     | 2.28 | 13.68 ± 1.03          | 4.47 |
| K562           | 5.64 ± 1.40|               |      |                       |      |
| K562/ADR       | 12.86 ± 1.97|               |      |                       |      |

RNA using oligo(dT) priming (Thermoscript RT kit; Invitrogen), followed by SYBR-Green real-time PCR. The primers are as follows: MDR1, 5′-ctcagtgttgcctggttg-3′, and 5′-gcttctttttgctgtg-3′; GAPDH, 5′-gatctacgggatggcttg-3′, 5′-ggtatttggagggatatc-3′. The threshold cycle for PCR products was defined as the cycle at which the SYBR-Green fluorescent signal was 20 standard deviations above background. Relative quantification of gene transcription was performed using the comparative CT method (or DDCT, method available in the user bulletin of ABI Prism 7700 sequence detection system) with GAPDH and β-actin as the control. Melting dissociation was performed to evaluate the purity of the PCR product.

2.6. Drug Efflux Assay. KB and KB/VCR cell lines were cultured at 37°C in a 5% CO2 atmosphere. After treating with YQ36(10 μM) or doxorubicin(10 μM) for 3 hours, cells were washed twice with serum-free media, then incubated in fresh media plus 10% heat-inactivated newborn calf serum; cells were harvested at different times (0, 15, 30, 45, 60, 120, 180, 240 minutes as indicated in Results) and checked for fluorescence using a FACS Calibur (Becton Dickinson, Lincoln Park, NJ). Excitation and emission wavelengths were 488 and 585 nm, respectively.

2.7. DNA Agarose Gel Electrophoresis. KB/VCR cells (5 × 105) were exposed to YQ36 for 48 hours. DNA fragmentation was extracted as in a previous report [11]. Briefly, harvested cells were lysed by equal volumes of 1.2% SDS. By adding 7/10 volume of the precipitation solution (3 mol/L CsCl, 1 mol/L potassium acetate, 0.67 mol/L acetic acid) and spinning for 15 minutes at 14,000 g, DNA fragmentation was kept in the supernatant, which was then absorbed by a miniprep spin column. Finally, DNA was eluted with 50 μL TE buffer (pH, 8.0) and electrophoresed in parallel on the same 1.5% agarose gel in 1x TAE buffer with 0.5 mg/mL ethidium bromide staining to facilitate visualization by fluorescence under UV light. Images were photographed by Bio-Rad GD2000 (Bio-Rad, Hercules, CA, USA).

2.8. Mitochondrial Isolation. Harvested cells were washed with cold PBS and resuspend in 200 μL mitochondrial isolation buffer (Mannitol 200 mM, Sucrose 70 mM, EGTA 1 mM, HEPES 10 mM, Protease inhibitor cocktail). Homogenize cells on ice with a glass homogenizer. Centrifuge 1000 g at 4°C for 10 minutes and collect the supernatant. Then Spin 10,000–15,000 g for 15 minutes at 4°C and the pellet was collected for further experiment.

2.9. Caspase-3/7 Activity and PKC Activity Assay. Cells were cultured in 96-well plates, and caspase activity was measured using the luminescent Caspase-Glo 3/7 Assay (Promega, Madison, WI), following the manufacturer’s protocol at the indicated time points. PKC activity assay was measured using the Pep Tag Non-Radioactive PKC Assay (Promega, Madison, WI) following the manufacturers protocol.

2.10. P-gp ATPase Assays. P-gp ATPase assay was conducted using the P-gp Glo Assay System (Promega, Madison, WI), following the manufacturers protocol. The activity of Pgp ATPase was measured in the presence of variation concentration Na3VO4 and YQ36. The luminescence of the sample reflects the ATP level in the sample, which is negatively correlated with the activity of Pgp ATPase and was detected in a Varioskan Flash (Thermo Type3001).

2.11. Western Analysis. Proteins of KB and KB/VCR cell lines were extracted in immunoprecipitation assay buffer (50.0 mM NaCl, 50.0 mM Tris–HCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS) and 20.0–100.0 μg of total protein was loaded per lane. Proteins were fractionated on 8%–15% Tris-Glycine pre-cast gels, transferred to nitrocellulose membrane (Pierce Biotechnology, Rockford, IL), and probed with primary antibodies and then HRP-labeled secondary antibodies. Proteins were visualized using ECL (Pierce Biotechnology).

2.12. Statistical Analysis. For all parameters measured, the values for all samples in different experimental conditions were averaged and the SD of the mean was calculated. The significance of differences between the values of the groups was determined with unpaired Student’s t-test. Significance levels were set at P < .05.

3. Results

3.1. Antiproliferation of YQ36 on Sensitive and Multidrug-Resistance Cells. In order to evaluate the anti-multidrug resistant effect of YQ36 on tumor cells, antiproliferative activity of YQ36 was tested using a panel of chemosensitive
cell lines and their chemoresistant sublines, including human oral squamous carcinoma cell lines (KB and KB/VCR), human breast cancer cell lines (MCF-7 and MCF-7/DOR), and human leukemia cell lines (K562 and K562/ADR). All the six cell lines responded dose dependently to YQ36 and Doxorubicin (DOX) after 48 hours treatment. The IC50 values (Table 1) showed that, compared with their parental cells, KB/VCR, MCF-7/DOR, and K562/ADR cells appeared to be more resistant to DOX (6.60-fold, 4.49-fold, and 4.47-fold, resp.). In contrast, YQ36 showed parallel cytotoxicity on KB, MCF-7, and K562 cells (IC50 = 5.80, 15.68, and 5.64 μM) and their corresponding MDR-positive subcells (IC50 = 4.78, 7.90, and 12.86 μM). The dose-response curves of all six cell lines were shown in Figure 2. By comparing IC50 values, the human oral squamous carcinoma cell lines were chosen for further research. To sum up, YQ36 showed strong cytotoxic effect on both parental and MDR cell lines, suggesting that YQ36 might have promising anti-multidrug resistant activity.

3.2. MDR1 Was Amplified and P-gp Was Expressed in KB/VCR Cells. To characterize the drug-resistant cells, we used Real-Time PCR to identify mRNAs which were differentially expressed in parental and daughter cell lines. Figure 3(a) showed that MDR1 was dramatically overexpressed in drug-resistant cells and it showed a 107-fold at MDR1 mRNA level in KB/VCR cells compared with that of KB cells. In addition, Western Blotting results confirmed P-gp expression in KB/VCR cells only (Figure 3(a)). These results suggested that MDR1 (P-gp expression) is involved in the acquired resistance as previously reported.

3.3. YQ36 Could Accumulate in KB/VCR Cells. The major function of P-gp was to increase drug efflux, which is one of the most important mechanisms of MDR to decrease intracellular drug concentration. In our study, as shown in Figure 3(b), the amount of YQ36 was not obviously decreased in KB and KB/VCR cells. However, the amount of doxorubicin (DOX) was decreased significantly in drug-resistant cells in a time-dependent manner. These results indicated that YQ36, rather than Dox, was accumulated in P-gp positive KB/VCR cells, which may contribute to exhibited equal effect of YQ36 in both parental and MDR cell lines.

3.4. YQ36 Had No Effects on P-gp Expression or Activity. YQ36 accumulated in KB/VCR cells might be attributed to the modulation of P-gp function. There are two potential mechanisms accounting for the decreasing of P-gp function: first, downregulated expression of P-gp; second, inactivate the function of P-gp. In order to determine whether YQ36 was capable of reducing the expression of P-gp, we detected P-gp expression in KB/VCR cells treated with 10 μM YQ36 for 12, 24, and 48 hours. The results displayed that P-gp level was not obviously decreased (Figure 3(c)).

There were some evidences indicated that Protein Kinase C (PKC) could phosphorylate P-gp and might change its activity [4, 12, 13]. It was also reported that BIM analogues had strong inhibition on PKC activity. As a BIM analogue, YQ36 might inactivate P-gp via reduced phosphorylation by inhibiting PKC. To examine this possibility, we detected the effect of YQ36 on PKC by Peptag- Assay Kit. According to the manufacturer’s protocol, the level of phosphorylated specific peptide indicated the activity of pan-PKC. As shown in Figure 3(d), YQ36 failed to decrease the amount of phosphorylated specific peptide. However, the positive control group BIM exhibited inhibition of PKC activity in a dose-dependent manner. These results indicated that YQ36, as one of the analogues of BIM, had little effect on the activity of PKC, implying that YQ36 might not be able to inhibit the phosphorylation of P-gp thus fail to affect the function of P-gp.

As YQ36 had no effect on P-gp expression or activity, one more explanation for accumulation of YQ36 in KB/VCR cells might be that YQ36 did not serve as a substrate for P-gp, thus escaping from effluxed mediated by P-gp. In order to verify this hypothesis, Pgp-Glo Assay system was used to detect whether YQ36 was the substrate for human recombination P-gp. The results came out that YQ36 failed to disturb the function of ATPase in human recombination P-gp. In contrast, sodium vanadate exhibited dose-dependent inhibition on activity of human recombination P-gp in vitro (Figure 3(e)). To summarize, YQ36 might not be the substrate for P-gp. These results may explain for the fact that YQ36 could be accumulated in P-gp positive cells.

3.5. YQ36 Caused Apoptosis in KB/VCR Cells In Vitro. DNA fragmentation is a common feature of apoptotic cell death. Electrophoretic analysis of DNA (Figure 4(a)) showed that exposure of KB/VCR cells to 10 μM YQ36 for 12, 24, and 48 hours resulted in a characteristic fragmentation of DNA, a biochemical hallmark of apoptosis, at intranucleosomal sites. In addition, the result of measuring caspase-3/7 activity also indicated that YQ36 induced apoptosis in KB/VCR cells in a time-dependent manner (Figure 4(b)).

Caspase, known as crucial mediators of apoptosis, depends on proteolytic activation of the procaspase forms to enzymatically active forms. When KB/VCR cells were treated with 10 μM YQ36 for 12, 24, and 48 hours, the levels of cleaved caspase3 and Apaf-1 were significantly increased accompanied by the downregulation of both procaspase-9 and procaspase-3 (Figure 4(c)). In order to elucidate whether YQ36-driven apoptosis was accompanied by caspase-independent apoptosis or necrosis, KB/VCR cells were pretreated with the caspase inhibitor BOC-D-FMK (100 μM) and Z-DEVD-FMK (40 μM), respectively, followed by exposure to 10 μM YQ36 for 48 hours. As illustrated in Figures 5(a) and 5(b), the caspase inhibitor BOC-D-FMK (100 μM) and Z-DEVD-FMK (40 μM) significantly reversed YQ36-induced death. Collectively, the main mechanism of YQ36 cytotoxicity is based on caspase-dependent apoptosis induction.

There are many proteins related to the mitochondrial pathway. MAPks and Bcl-2 family were two major protein families regulating mitochondria and caspase-3 pathway. The expressions of Bcl-2, Bax, JNK, and p-JNK were measured in KB/VCR cells treated with YQ36 (10 μM, 12–48 hours). As shown in Figure 4(c), YQ36 increased the expression of total Bcl-2, Bax, JNK, and p-JNK.
Figure 2: YQ36 inhibited the proliferation on three panels of chemosensitive tumor cells and their chemoresistance subline. (a) Human oral squamous carcinoma cell lines (KB and KB/VCR) were treated with YQ36 or DOX (0.16–100 μM) for 48 hours. Points represent the mean fractional survival; error bars represent standard deviation. (b) Human breast cancer cell lines (MCF-7 and MCF-7/DOR) were treated with YQ36 or DOX (0.16–100 μM) for 48 hours. Points represent the mean fractional survival; error bars represent standard deviation. (c) Human leukemia cell lines (K562 and K562/ADR) were treated with YQ36 or DOX (0.32–100 μM) for 48 hours. Points represent the mean fractional survival; error bars represent standard deviation.

and phosphorylated JNK and decreased Bcl-2 expression in 48 hours. There was, thereby, an increase in Bax/Bcl-2 ratio after YQ36 treatment (Figure 4(f)). In order to further demonstrate that Bcl-2 family participated in YQ36-induced apoptosis, we examined the protein expression of Bax from the mitochondrial fraction. The data suggested that there was an increased amount of Bax in mitochondrial fraction (Figure 4(e)).
Figure 3: The action of YQ36-induced antimultidrug-resistance on KB/VCR cells.
Recent research demonstrated that resistance to chemotherapeutic drugs was associated with antiapoptotic proteins upregulation. To explore whether the antiapoptotic proteins play an important role in YQ36’s anti-multidrug resistant mechanism in KB/VCR cells, the expressions of Akt, p-Akt, Smac, and XIAP were measured by using Western blotting. As shown in Figure 4(d), YQ36 (10 μM) obviously decreased XIAP protein levels (12–48 hours), whereas, the expression of Smac was not changed obviously, and the decrease of p-Akt (Ser473) was not reduced until exposure time prolonged to 48 hours.

3.6. YQ36 Accumulated in Nucleus and Induced DNA Damage in KB/VCR Cells. Interestingly, YQ36 was a fluorescent compound; so we further investigated the subcellular distribution of YQ36. Fluorescence Microscope was used to observe the distribution of YQ36 in KB/VCR cells. As shown in Figure 6(a), YQ36 mainly accumulated in the nucleus (arrow in Figure 6(a)). This observation encouraged us to further investigate the action of YQ36 in the nucleus.

Mounting evidences indicated that p53 was a sense element in DNA damage. To determine whether YQ36 induced DNA damage, p53 and p-p53 (Ser20) were detected.
Figure 5: YQ36-caused apoptosis was caspase-dependent. (a) The morphology of KB/VCR cells treated with YQ36 (10 μM, 48 hours) plus with or without caspase inhibitor (100 μM BOC-D-FMK and 40 μM Z-DEVD-FMK) (100×). (b) The survival rate of KB/VCR cells treated with YQ36 (10 μM, 48 hours) plus with or without caspase inhibitor (100 μM BOC-D-FMK and 40 μM Z-DEVD-FMK).

by Western blotting. As shown in Figure 6(b), the phosphorylation level of p53 increased considerably while the expression of total p53 remained unchanged. These results suggested that YQ36 induced DNA damage in KB/VCR cells.

4. Discussion

Up to now, the escape of cancer cells from chemotherapy through activation of MDR mechanisms was a major reason for systemic cancer treatment failure. Among these, the effectiveness of many clinical used drugs is limited by the fact that they are substrates of the efflux pumps P-gp (MDR1). In this study, YQ36 exhibited high anti-multidrugresistant activity in P-gp-positive cancer cells, causing DNA damage and finally induced apoptosis via mitochondrial pathway in KB/VCR cells.

So far, numerous studies have confirmed that the expression of P-gp is an adverse prognostic factor for complete remission and survival in both leukemia and solid tumor [14, 15]. P-gp, a member of the ABCB subfamily, stands out among ABC transporters by conferring the strongest resistance to the widest variety of compounds. Three-dimensional modeling of human MDR1/P-gp indicates that these glycoproteins function as efficient ATP-dependent gate-keepers, which scan the plasma membrane and its inner leaflet to flip lipophilic substrates to the outer membrane leaflet [16]. Several agents with hyperactivity against MDR cells, as, for example, doxorubicin [17], were P-gp transport substrates. In our experiment, similar intracellular concentrations of YQ36 were observed in KB/VCR cells and KB cells. In addition, it showed that neither the P-gp protein level nor the phosphorylation of P-gp was downregulated in MDR-positive cells. It indicated that YQ36 may not be a P-gp transport substrate. This hypothesis was further confirmed by the results that YQ36 failed to modify the function of ATPase in human recombination P-gp.

Most of studies had described that increased expression and activity of PKC could be associated with the MDR phenotype [12, 13]. The importance of PKC-mediated phosphorylation of P-gp is still unclear in affecting P-gp function. Some BIM analogues, as the PKC inhibitor, had been reported to reverse multidrug resistance in MDR-positive cells [7, 18, 19]. Interestingly, some BIM analogues (such as Ro 32-2241 and GF 109203X) were the substrates for P-gp but did not reverse multidrug resistance by inhibiting PKC phosphorylation of the P-gp to modulate P-gp function [7, 19]. Thus the escape from being substrates for P-gp, rather than the inhibition of PKC-dependent phosphorylation of the P-gp, might be the key point of reversing multidrug resistance. In this study, our findings suggested that YQ36, a novel BIM analogue, was not the substrate for P-gp and exhibited high activity in both parental cells and MDR cells. It is the first time to report that BIM derivates may be an effective anti-multidrug-resistance compound without inhibiting the activity of PKC.

It was most likely that the antitumor effect on KB/VCR cells was based on YQ36-mediated apoptosis. The results of the electrophoretic analysis of DNA and caspase-3/7 activity were consistent with our hypothesis. Moreover, Western blotting results suggested that the YQ36 treatment induced apoptosis via mitochondrial pathway. Mitochondria play a pivotal role in the regulation of apoptosis induced by diverse death stimuli [20–22]. Once transition pores opened on mitochondria, cytochrome c, the cell death-promoting factor, released and leads to the formation of a complex consisting of apoptosis-activating factor 1 (Apaf-1) and caspase-9 and initiated the proteolytic cascade. Therefore, the observation of YQ36-mediated activation of procaspase-9, procaspase-3 suggested that mitochondrial-mediated caspase cascade pathway played a pivotal role in YQ36-induced apoptosis. Furthermore, YQ36-induced downregulation of XIAP, one of the inhibitors of apoptosis proteins (IAPs) family [23, 24], was detected in KB/VCR cells. Since XIAP was able to inhibit activation of caspase-3 to protect cells from apoptosis, downregulation of XIAP provided an additional
indication that YQ36-exerted antitumor effect was related to the activation of caspase cascade.

There are two major pathways involved in mitochondria-mediated apoptosis. Through convergence of the signaling at the mitochondrial membrane, Bcl-2 family [25, 26] can influence the mitochondria directly. The Bcl-2 protein family includes proapoptotic members, such as Bcl-2, Bcl-xl, and Bcl-W and antiapoptotic members like Bax. Antia apoptotic proteins act as repressors of apoptosis by blocking the release of cytochrome c, whereas proapoptotic members act as promoters. The overall action was dependent on the balance between Bcl-2 [27, 28] and Bax [29], and the increase ratio of Bax/Bcl-2 was associated with the release of cytochrome c [30]. Our results demonstrated a dramatic increase of Bax/Bcl-2 ratio in KB/VCR cells treated with YQ36, indicating that the regulation of Bcl-2 family expression plays an important role in YQ36-induced apoptosis. It is reported that JNK can influence the binding ability of Bcl-2 with Bax and impair antiapoptotic function of Bcl-2 by phosphorylation [31, 32]. In present study, our results exhibited that YQ36 obviously upregulated both the total expression of JNK and its phosphorylated status. These observations suggested that the MAPK’s activity may be also involved in the YQ36-mediated apoptotic process.

Recent investigations had demonstrated that the coordinated overexpression of the different IAPs may affect the tumor cell resistance to drug-induced apoptosis [33]. The IAPs are a recently identified group of antiapoptotic proteins, including XIAP, AKT, and others. XIAP expression had been demonstrated to be associated with a shorter duration of remission and overall survival in acute myeloid leukemia [34]. In contrast, Smac/Diablo is a mitochondrial protein that functions as a regulatory component during apoptosis [35, 36]. The interaction of Smac/Diablo with XIAP could relieve the inhibitory effect of IAPs on caspase [35, 36]. Our results showed that YQ36 obviously downregulated the expression of XIAP but failed in changing the level of Smac/Diablo, indicating that the regulation of XIAP expression might play an important role in YQ36-induced apoptosis in KB/VCR cells.

The major function of tumor suppressor protein p53 is to respond to DNA damage. The phosphorylated p53 promoted activation of p53 in response to DNA damage [9, 37]. In our study, YQ36-caused DNA damage was confirmed by detected upregulation of p-p53 (Ser20) before changing the expression of downstream proteins (such as JNK, Bcl-2, caspase). Moreover, recent research demonstrates that p53, as a transcriptional factor, mediates the upregulation of Bax after its activation. However, our result showed the whole protein expression of Bax was rarely changed. It might implicated that it was the MAPK signaling rather than the p53 signaling regulating the Bcl-2 family protein function. Collectively, these results indicated that YQ36-elicted apoptosis of KB/VCR cells was primarily caused by DNA damage. Although we failed to distinguish the type of DNA damage, according to our data, YQ36-caused DNA damage might not be generated from the DNA cross-link or double strand breaks mediated by topo II (data not shown). Further experiments were still undergoing to reveal the exact style of YQ36-induced DNA damage.

5. Conclusions

Taken together, our experiments demonstrated that YQ36 was a potent agent against MDR cells and DNA damage together with mitochondrial pathway might be involved in signaling YQ36-induced apoptosis.

Acknowledgments

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