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Q39, a novel synthetic Quinoxaline 1,4-Di-*N*-oxide compound with anti-cancer activity in hypoxia ☆

Qinjie Weng [a], Duoduo Wang [a], Peng Guo [b], Liang Fang [a], Yongzhou Hu [b], Qiaojun He [a,*], Bo Yang [a,*]

[a] *Institute of Pharmacology & Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*
[b] *Zhejiang University-Ecole Normole Superienre Joint Laboratory Medicinal Chemistry, Zhejiang University, Hangzhou 310058, China*

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Abstract

Hypoxia is one of the inevitable circumstances in various tumors and results in tumor resistance to radiotherapy and chemotherapy. The present data showed that 3-(4-bromophenyl)-2-(ethylsulfonyl)-6-methylquinoxaline 1,4-dioxide (Q39), derived from Quinoxaline 1,4-Di-*N*-oxide, possessed high anti-cancer activity in hypoxia. Cytotoxicity assay demonstrated that Q39 is a potential and high efficient anti-cancer compound in all tested cell lines with IC_{50} values of 0.18±0.03–8.88±1.12 μM in hypoxia and 0.33±0.04–8.74±1.28 μM in normoxia . In the following work concerning the mechanism of Q39 in hypoxia, we confirmed that Q39 could cause the apoptosis of K562 cells in a time-dependent manner. By fluorescence stain assay, Q39-induced mitochondria membrane potential ($\Delta\Psi_m$) loss was observed in K562 cells in hypoxia. Based on the western blotting, Q39 decreased the protein expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) in hypoxia. The compound caused the activation of caspase-3 and subsequent cleavage of its substrate poly (ADP-ribose) polymerase (PARP) in hypoxia. Meanwhile, we found the upregulation of Bax by Q39 in K562 cells as well as the downregulation of Bcl-2. Q39 also influenced the expression of Mitogen-Activated Protein Kinase (MAPKs) and other proteins relative to mitochondria induced apoptosis. In addition, Q39-mediated apoptosis was not reversed after treatment with the JNK-specific inhibitor. In summary, the present study demonstrated Q39 was a novel compound against cancer cells in hypoxia. The mitochondrial pathway mediated by Bcl-2 protein family and MAPKs and the HIF-1 pathway might be involved in signaling Q39-induced apoptosis.

Keywords: Hypoxia; HIF-1α; Bcl-2 protein family; MAPK; Mitochondria

1. Introduction

A different level of regulation in tumor progression is controlled by the response of tumor to hypoxic condition. Hypoxia has been shown to favor the self-renewal of murine and human hematopoietic stem cells (Ivanovic et al., 2002). Chronic myeloid leukemia is a hematopoietic stem cell malignancy, which originates from bone marrow, a hypoxic tissue compartment. It has been reported that hypoxia has a greater relevance in chronic myeloid leukemia and there is an antagonistic effect of hypoxia on many chemotherapeutic agents (Desplat et al., 2002; Aichberger et al., 2005). Hypoxia-inducible factor-1 (HIF-1) is a transcriptional complex that is activated in response to hypoxia and other growth factors. HIF-1 plays a central role in tumor progression, invasion and metastasis.

Overexpression of the HIF-1α subunit has been observed in many human cancers, which is associated with a poor prognostic outcome in conventional treatments. It is also well established that HIF-1 activation stimulates angiogenesis in mature tumors by upregulating multiple proangiogenic factors, including vascular

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* Corresponding authors. 388# Yuhangtang Road, Institute of Pharmacology & Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China. Tel./fax: +86 571 88208400.
E-mail addresses: qiaojunhe@zju.edu.cn (Q. He), yang924@zju.edu.cn (B. Yang).

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endothelial growth factor (VEGF). Therefore, targeting HIF-1α using novel small molecular inhibitors is an attractive strategy for therapeutic development (Semenza, 2003).

Tirapazamine (TPZ) has been reported as one of the most potent anti-tumor compounds in hypoxia. Because of its simple structure and strong hypoxic cytotoxicity against a variety of human cancer cells, numerous studies on the structure–activity relationship of TPZ have demonstrated the importance of the *N*-oxide group for the selective activity and suggested that the more negative the reduction potential the greater the hypoxia selectivity, to the point at which enzymes can no longer reduce the compound (Monge et al., 1995a,b). 3-(4-Bromophenyl)-2-(ethylsulfonyl)-6-methylquinoxaline 1,4-dioxide (Q39, Fig. 1), one of the synthesized TPZ analogues, can induce apoptosis in cancer cells, showing great antiproliferative action *in vitro*, therefore, we are interested in exploring the anti-cancer molecular mechanism of Q39 in hypoxia.

Recent investigations have indicated that the mitochondrial dysfunction is involved in apoptosis (Tang and Zhang, 2005). A consequent loss of mitochondrial membrane potential (Rotem et al., 2005; Ogbourne et al., 2004) is reported as typical phenomena in the process of apoptosis related to mitochondria (Wu et al., 2005; Doi et al., 2004; Choi et al., 2002; Ghobrial et al., 2005; Tang and Zhang, 2005). It is known that mitochondria-mediated apoptosis is regulated by two major pathways. The death receptors on the cell surface can affect the mitochondria indirectly by regulation of MAPKs and the convergence of the signaling at the mitochondria can influence the mitochondria directly by regulation of Bcl-2 protein family.

In this article, we reported the antiproliferative activity of Q39 on five cancer cell lines in hypoxia. In addition, the involvement of mitochondrial dysfunction and HIF-1α signaling pathway in Q39-induced apoptosis was investigated.

2. Materials and methods

2.1. Drugs and chemicals

Q39 was supplied by professor Yongzhou Hu. Q39's yield was 84.9% and its general synthesis process was listed in Fig. 1. The structures of Q39 were determined by IR, MS, and NMR. Column chromatography was carried out with silica gel 60 Merck for purification, and the purity of Q39 was >99%. Stock solution of Q39 (25 mM) was prepared with dimethyl sulfoxide (DMSO) and stored at −20 °C for *in vitro* test. The stock solution was further diluted with the appropriate assay medium immediately before use. The mitochondrial fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). Stock solutions of JC-1 (2.0 mg/ml) were dissolved in DMSO and stored at −20 °C. The primary antibodies to procaspase-3, procaspase-9, Bcl-2, Bax, PARP, extracellular signal-regulated kinase (ERK1/2), p38, c-Jun NH2-terminal protein kinase (JNK), p-ERK1/2, p-p38, p-JNK, x-linked inhibitor of apoptosis protein (XIAP), HIF-1α, VEGF, α-tubulin and β-actin, and HRP-labeled secondary anti-goat, anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL, a western blot detection reagent, was purchased from Amersham Biosciences (Piscataway, NJ).

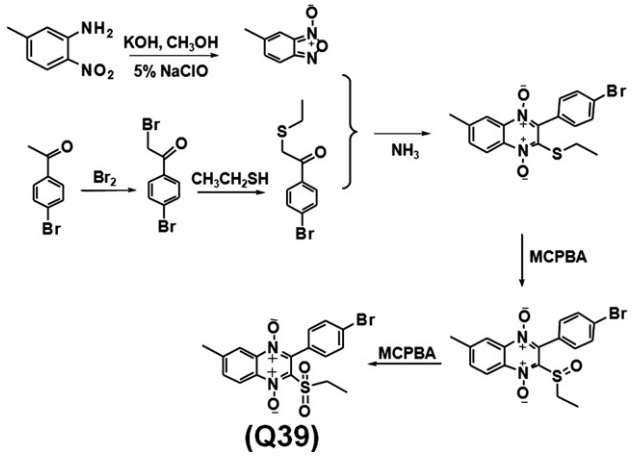

Fig. 1. Chemical structure and general synthesis process of Q39.

2.2. Cell lines and cell culture

Five human cancer cell lines were employed. Androgen-independent prostate carcinoma PC3, chronic myeloid leukemia K562, hepatocellular carcinoma SMMC-7721, human gastric carcinoma SGC-7901, and esophageal carcinoma ECA-109 cells were maintained in RPMI 1640. All media were supplemented with 10% FCS plus 2 mM glutamine and 50 unit/ml penicillin. All of the cell lines were purchased from the Institute of Cell Biology (Shanghai, China) and incubated at 37 °C in a 5% CO_2 atmosphere. Moderate hypoxic conditions (3% O_2) were established in a hypoxia incubator (Forma Scientific, Inc., Marietta, OH) where N_2 was used to compensate for the reduced O_2 level.

2.3. Cytotoxicity assay

Cancer cells were seeded in 96-well microtiter plates (4000 cells/well), and were cultured in normoxia and hypoxia. The hypoxic cells were allowed to attach 1 day prior to the addition of Q39 (0–25 μM) in complete medium (to various final concentrations in 200 μl of complete medium) in replicates of 4 wells per condition. Plates were assayed at 72 h after initiation of drug exposure. Afterwards, 10 μl of stock 3-[4,5-dimethylthia-zol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution was added to each well (0.5 mg/ml) for another 4 h incubation (37 °C, 5% CO_2). After 4 h incubation, 200 μl of DMSO was added to each well and optical density (OD) was read at 570 nm by Thermo Multiskan Spectrum (Thermo Electron Corporation). The IC_{50} values were calculated using the PrismPad computer program (GraphPad Software, Inc., San Diego, CA) and were defined as concentration of drug causing 50% inhibition in absorbance compared with control (vehicle) cells.

2.4. Apoptosis assay

The K562 cells were treated with Q39 and/or the general caspase inhibitor, z-VAD-fmk (R&D Systems, Inc., Minneapolis, MN, USA), and JNK-specific inhibitor, SP600125 (Calbiochem, San Diego, CA, USA), as follows: Q39 (6.0 μM) alone for 12–48 h in 3% O_2 or 20% O_2 respectively; Q39 (6.0 μM)+z-VAD-fmk (10.0 μM) and/or SP600125 (20.0 μM) for 48 h in hypoxia. Detection of apoptosis by FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ) was performed using the Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (BioVision, Mountain View, CA).

2.5. Assessment of mitochondrial membrane potential ($\Delta\Psi_m$) transition

The $\Delta\Psi_m$ was determined (Mancini et al., 1997) in K562 cells after 6–48 h treatment with 6.0 μM Q39 in 3% O_2. Afterward cells were collected and washed by PBS. Cells were suspended in 1 ml of complete medium containing 10 μg/ml of JC-1 for 30 min at 37 °C. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria (Maurer et al., 1999), indicated by a fluorescence emission shift from green to red. To assess mitochondrial potential transition, 10,000 cells/sample stained by JC-1 were observed by fluorescent microscope (Leica, Germane) and flow cytometry. Mitochondria depolarization was specifically indicated by a decrease in the red to green fluorescence intensity.

2.6. Protein expression

Proteins of K562 cells incubated with 6.0 μM Q39 for 0.5, 1, 2, 4, 8, 12 and 24 h (in 3% O_2) were extracted in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris, 2 mM EGTA, 2 mM EDTA, 25 mM NaF, 25 mM glycerophosphate, 0.2% Triton X-100, 0.3% NONIDET P-40, 0.1 mM PMSF). Total protein concentrations of whole cell lysates were determined using BioRad BCA method (PIERCE, Rockford, IL). Equal amounts of protein sampled from whole cell lysates were subjected to electrophoresis on 10%–12% Tris–Glycine pre-cast gels (Novex, San Diego, CA) and electroblotted onto Immobilon-P Transfer Membrane (Millipore Corporation, Billerica, Massachusetts), and probed with primary antibodies and then incubated with a horseradish peroxidase (HRP) conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescent (ECL) Western Blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

2.7. Assays of caspase-3, caspase-8 and caspase-9 activities

Caspase-3 activity was measured with use of the CaspACE Assay System Fluorometric Kit Caspase-Glo® 8 Assay, Caspase-Glo® 9 Assay (Promega Corporation, Madison, WI). Cells were initially seeded at a density of 1×10^6 in 100 mm^2 dishes. After treatment for the indicated time of Q39 (6.0 μM), caspase-3, caspase-8 and caspase-9 activities were measured according to manufacturer's instructions.

3. Results

3.1. Antiproliferative activity of Q39

Cytotoxicity assays of Q39 in hypoxia were determined on five human cancer cell lines, including K562, SMMC-7721, ECA-109, SGC-7901 and PC3. As shown in Table 1, Q39 exhibits antiproliferative activity on all five tested cell lines in a dose-dependent manner, and the IC_{50} values of Q39 for all five tumor cells are <10.0 μM in hypoxia and in normoxia. Although Q39 has little advantages against ECA-109 and SGC-7901 cells in hypoxia, it reveals hypoxic selectivity in the other three cell lines, suggesting that Q39 has promising anti-cancer activity among the specific cancer cells in hypoxia.

3.2. Q39-induced apoptosis

The K562 cells were administrated with 6.0 μM Q39 for 12, 24 and 48 h in hypoxia or in normoxia, and examined for apoptosis by Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA) and flow cytometry. Spontaneous apoptosis (control) was seen in 5.5% (Fig. 2B) of cells in normoxia and 4.1% in hypoxia (Fig. 2A). In normoxia, apoptosis was increased to 24.4% at 48 h, and the 12 h and 24 h groups didn't induce apoptosis (5.1% and 4.6%) relative to controls (Fig. 2B and D). In hypoxia, Q39 caused apoptosis in 34.5%, 42.5% and 60.1% of cells at 12, 24 and 48 h (Fig. 2A and D). Moreover, in the presence of pan-caspase inhibitor z-VAD-fmk (10.0 μM), Q39-induced apoptosis was significantly reduced from 60.1 to 23.2% at 48 h (Fig. 2C and E). In contrast to inhibition of caspase pathway, pretreatment with the JNK-specific inhibitor, SP600125, did not prevent Q39-induced cell death (Fig. 2F and G), indicating that Q39 induces apoptosis through caspase-dependent pathway and JNK activation is not involved in Q39-induced apoptosis.

3.3. Loss of mitochondrial membrane potential ($\Delta\Psi_m$)

Loss of $\Delta\Psi_m$ leads to release of cytochrome *C* from mitochondria and consequently trigger other apoptotic factors. We determined $\Delta\Psi_m$ in K562 cells administrated with 6.0 μM Q39 for 6–48 h in hypoxia. A loss of $\Delta\Psi_m$ decline was specifically indicated by a switch from the red to green fluorescence intensity. Compared with the corresponding control, Q39 obviously

Table 1
Cytotoxicity of Q39 on a panel of five human cancer cell lines

Original tumor type	Cell line	IC_{50} (μM)	
		3% O_2	20% O_2
Hepatoma	SMMC-7721	3.17±0.82	6.86±1.74
Chronic myelocytic leukemia	K562	0.18±0.03	0.33±0.04
Androgen-independent prostate cancer	PC3	4.98±0.56	8.74±1.28
Esophageal cancer	ECA-109	6.38±2.73	0.45±0.13
Gastric cancer	SGC-7901	8.88±1.12	6.75±2.33

Cells were exposed with various concentrations of Q39 for 72 h in hypoxia and in normoxia. Results were expressed as mean±SD.

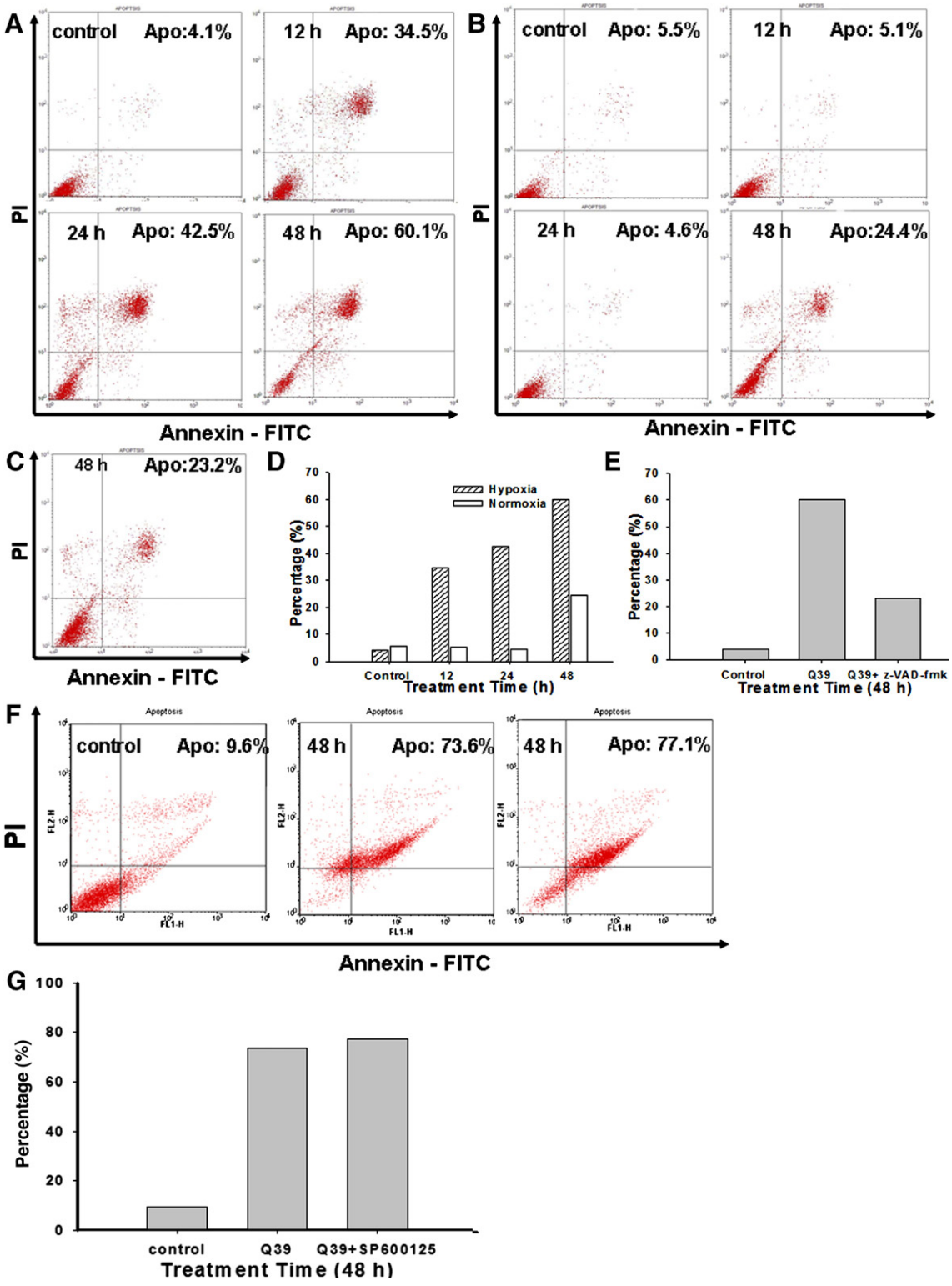

Fig. 2. Q39-induced apoptosis in K562 cells in hypoxia through caspase-dependent pathway. K562 cells were treated with Q39 (6.0 μM) for 12–48 h in 3% O_2 (A, D) and 20% O_2 (B, D) respectively , or Q39 (6.0 μM)+z-VAD-fmk (10.0 μM) and Q39+SP600125 (20.0 μM) for 48 h in 3% O_2 (C, E, F, G). After treatment, cells were harvested with Puck's EDTA, detection of apoptosis by flow cytometry was performed using the Annexin V/PI apoptosis detection kit. The DNA content of 15,000 events was analyzed by flow cytometry.

decreased $\Delta\Psi_m$ in K562 cells after 12–48 h incubation in a time-dependent manner (Fig. 3A). The quantitative data from flow cytometry was represented in Fig. 3B, which showed that the percentages of cells with low $\Delta\Psi_m$ were nearly 10-folds increase in the cells exposed to Q39 for 48 h compared with the corresponding control in hypoxia.

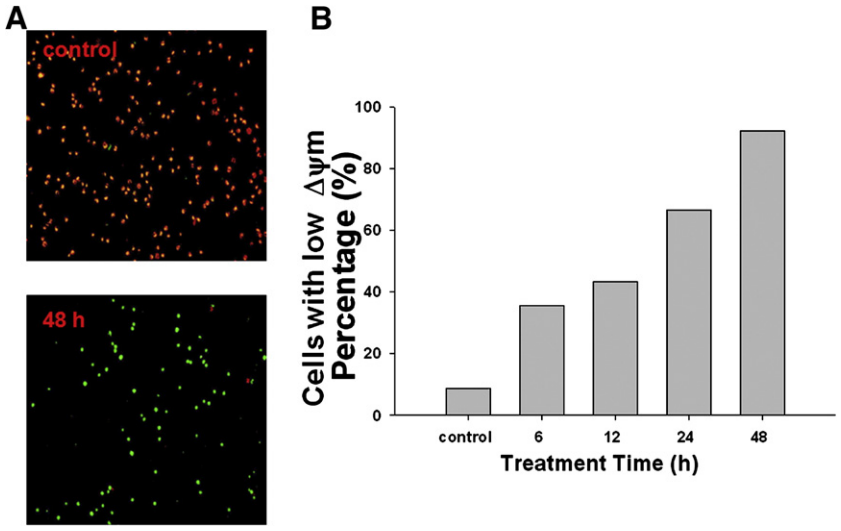

Fig. 3. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) induced by Q39. Cells were incubated in hypoxic condition, and were treated with Q39 (6.0 μM) for 6–48 h. To assess mitochondrial potential transition, 10,000 cells/sample stained by JC-1 were observed by fluorescent microscope (A) and flow cytometry (B).

3.4. Changes of protein expression related to mitochondria

It was reported that MAPKs and Bcl-2 family can be activated after mitochondrial dysfunction. K562 cells were preincubated overnight in serum-free medium, to confirm whether Q39 change protein expression related to mitochondria, the expression of ERK1/2, P38, JNK, p-ERK1/2, p-p38, p-JNK was measured in K562 cells treated with Q39 (6.0 μM, 0.5–24 h). As shown in Fig. 6B, Q39 (>2 h treatment) obviously decreases p-ERK and p-p38 phosphorylation status, meanwhile increases the expression of p-JNK in a time-dependent manner. Moreover, we detect the protein expression of Bcl-2 family, and we observed that Q39 treatment resulted in the decrease of Bcl-2 and increase of Bax (Fig. 4A). To clarify the role of JNK activation, we tested the effect of the specific JNK inhibitor, SP600125, on JNK activation and apoptosis induction by Q39. Pretreatment with SP600125 resulted in an inhibition of Q39-induced JNK activation. However, it failed to reverse Q39-mediated bcl-2 protein decline, and also failed to influence the elevated protein expression of cleaved caspase-3 induced by Q39 (Fig. 5). Since the blockage of JNK activation with SP600125 abolished caspase-3 activation, the result which the protein expression of caspase-3 was not downregulated suggested JNK activation may not directly involve in Q39-triggering apoptosis of K562 cells.

3.5. Caspase cascade in Q39-induced apoptosis

In most cases, the activation of caspase cascade accompanies with the apoptosis. The expression of procaspase-3, procaspase-9, XIAP and PARP was measured in K562 treated with Q39 (6.0 μM, 0.5–24 h). As shown in Fig. 4A, Q39 obviously decreases the protein levels of procaspase-3, procaspase-9 and

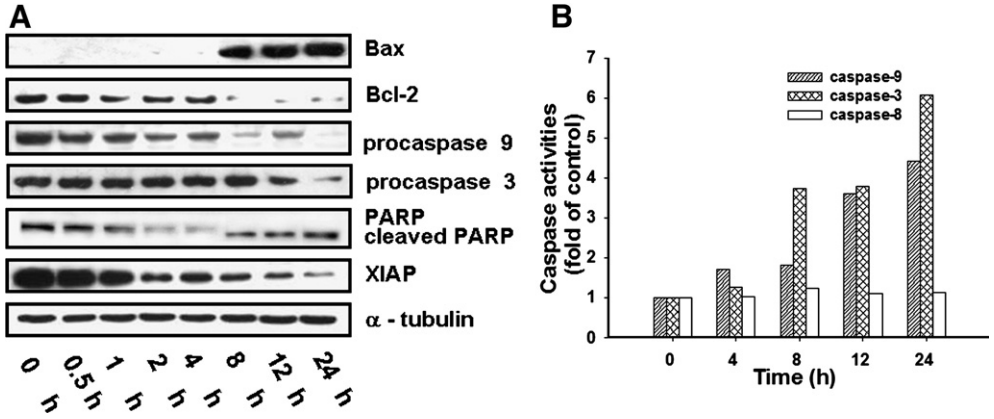

Fig. 4. Effect of Q39 on mitochondria related protein and activity of caspase-3 in K562 cells. (A) Cells were incubated in hypoxic condition, and were treated with Q39 (6.0 μM) for 0.5–24 h. Equal Amounts (40 μg/lane) of cellular protein were fractionated on Tris–Glycine pre-cast gels and electroblotted onto Immobilon-P Transfer Membrane, followed by immunoblotting with anti-Bcl-2, Bax, procaspase-3, procaspase-9, PARP, and XIAP antibodies. (B) Q39 regulates caspase-3, caspase-8 and caspase-9 activities in K562 cells. Cells were treated with 6.0 μM of Q39 for the indicated time and caspase-3, caspase-8 and caspase-9 activities were analyzed using specific kits. Each point represents the mean±SD.

XIAP, and induces the cleavage of PARP in a time-dependent manner. As shown in Fig. 4B, treatment of K562 cells with 6 μM of Q39 resulted in the detection of caspase-9 and caspase-3 activation as early as 4 h, whereas caspase-8 did not show activation.

3.6. Effects of Q39 on HIF-1α and VEGF protein levels

To determine whether (Q39) could decrease HIF-1α and VEGF protein levels, K562 cells were incubated with 6.0 μM of Q39 for 0.5–24 h in hypoxia. Western blot result showed that Q39 cause a decreased trend of HIF-1α and VEGF protein expression in hypoxia. Of interesting, only at 12 h, a relative elevated protein level of HIF-1α was observed (Fig. 6A).

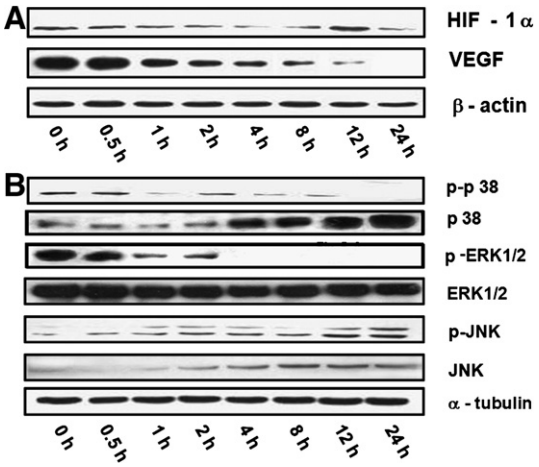

Fig. 6. Effect of Q39 on HIF-1α pathway related protein expression in K562 cells. Cells were incubated in hypoxic condition, and were treated with Q39 (6.0 μM) for 0.5–24 h. Equal amounts (40 μg/lane) of cellular protein were fractionated on Tris–Glycine pre-cast gels and electroblotted onto Immobilon-P Transfer Membrane, followed by immunoblotting with anti-HIF-1α, VEGF (A) and MAPKs, tubulin (B) antibodies.

4. Discussion

TPZ is a bioreductive drug that exhibits a high degree of selective toxicity toward hypoxic cells, and at doses that are used clinically, little or no cell killing is observed in aerobic cells (Wouters et al., 2001). Q39, modified from TPZ, belongs to a family of Quinoxaline 1,4-Di-*N*-oxides analogs. After modification, Q39's structure was fundamentally changed. As a novel compound, this paper focuses on the effects and mechanisms of its anti-cancer characteristics in hypoxia. The cytotoxic analysis *in vitro* indicates that Q39 is able to inhibit the cell proliferation on all five tested cancer cell lines both in hypoxia and normoxia. Particularly, Q39 shows stronger cytotoxic effect on K562, SMMC-7721 and PC3 cells but not SGC-7901 and ECA-109 cells in hypoxia than in normoxia, suggesting that it may serve as a potential hypoxic-selective compound in specific cancer cell lines.

In this study, we employed chronic myeloid leukemia K562 cells, which originate from bone marrow, to explore the anti-cancer mechanism of Q39 in hypoxia. The present study showed that Q39 could cause apoptosis and mitochondria depolarization in K562 cells in hypoxia (Figs. 2 and 3). It has been reported that mitochondria are involved in signaling apoptosis (Tang and Zhang, 2005), and $\Delta\Psi_m$ loss can induce the opening of permeability transition pores in mitochondria and release of cell death-promoting factor, such as cytochrome *C* (Ogbourne et al., 2004;Guo et al., 2005), which leads to the formation of a complex

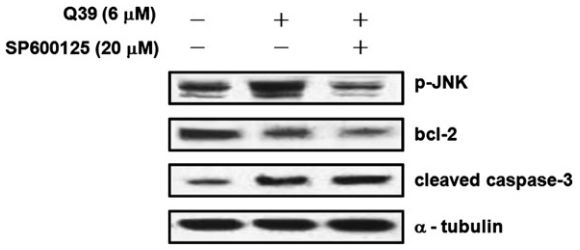

Fig. 5. Immunoblot analyses for p-JNK, bcl-2 and cleaved caspase-3 by JNK inhibitor SP600125 treatment. Cells were incubated with Q39 (6.0 μM) for 24 h, in the presence or absence of JNK inhibitor SP600125. Equal amounts of whole cell lysates (40 μg) were subjected to western blot analysis with respective antibodies. Similar patterns of protein expression were obtained from three independent experiments.

consisting of apoptosis-activating factor 1 (Apaf-1) and caspase-9, then initiates the proteolytic apoptotic cascade. Therefore, Q39-mediated procaspase-9 and -3 processing and subsequent cleavage of caspase-3's substrate PARP suggested that mitochondrial pathway played a very important role in Q39-induced apoptosis. Since the activation of caspase-3 is inhibited by members of the inhibitors of apoptosis proteins (IAPs) family (Miranda et al., 2003) at the downstream of the mitochondria-mediated pathway, the downregulation of XIAP (one member of the IAPs) provides an additional documentation that Q39-induced anti-leukemia is related to activate caspase cascade in hypoxia (Fig. 4A). Furthermore, the findings that Q39 activated the activity of caspase-3 and caspase-9 (Fig. 4B) and that the caspase-general inhibitor, z-VAD-fmk, abrogated apoptosis induced by Q39 (Fig. 2) indicated that caspase cascade activation is required for Q39-induced apoptosis in K562 cells in hypoxia.

JNK has been identified to contribute to death receptor transcription-dependent apoptotic signaling via c-Jun/AP-1, leading to transcriptional activation of FasL (Minden and Karin, 1997; Luo et al., 2003), and the blockage of JNK activation with JNK inhibitor (SP600125) abolishes caspase-8 and caspase-3 activations. In this study, the Q39-caused elevation in the expression levels of caspase-3 and Q39-induced apoptosis was not abrogated by the addition of JNK inhibitor (SP600125) in K562 cells (Figs. 5 and 2). Moreover, Q39 could not activate caspase-8 activity. These results that highlight death receptor signaling pathway may not participate in Q39-induced apoptosis. Combination with evidence of mitochondria depolarization and the activation of caspase-9 and caspase-3, we presume that mitochondrial permeability changes mediated mechanism by which Q39-triggered apoptosis in K562 cells.

According to the recent investigations, two major pathways are relevant with mitochondria-mediated apoptosis. Through convergence of the signaling at the mitochondrial membrane, Bcl-2 protein family, which includes pro-apoptotic members, such as Bax, Bad and Bcl-Xs, and anti-apoptotic members, such

as Bcl-2, Bcl-XL and Bcl-W, can influence the mitochondria directly. Anti-apoptotic members act as repressors of apoptosis by blocking the release of cytochrome *c*, whereas pro-apoptotic members act as promoters. The final action depends on the balance between Bcl-2 (Yamanaka et al., 2005; Sinicrope and Penington, 2005) and Bax (Hayward et al., 2004), and the increase of Bax and decrease of Bcl-2 could induce the release of cytochrome *C* (Katiyar et al., 2005). Our results demonstrate a dramatic increase of Bax and decrease of Bcl-2 in K562 cells treated with Q39, indicating that the regulation of Bcl-2 protein family expression does contribute to the Q39-induced apoptosis.

MAPKs, a protein family related to death receptors on the cell surface, can indirectly regulate the apoptosis of cancer cells. The MAPK family consists of a superfamily of three parallel signal transduction modules converging on the serine/threonine kinases JNK, p38 and ERK (Chang and Karin, 2001; Nozaki et al., 2001). These kinases are activated by a variety of stimuli and are intimately involved in diverse cellular processes including responses to DNA damage or osmotic shock, mitogenic stimuli, cell differentiation and survival (Cobb, 1999). Although JNK is known to mediate Fas-induced apoptosis in neuronal cells (Le-Niculescu et al., 1999), it can also be beneficial by interrupting p38 kinase and preventing cell death after tumor necrosis factor-α exposure in cardiomyocytes (Minamino et al., 1999). Similarly, both deleterious and beneficial actions of p38 signaling have been documented (Lee and Lo, 2003). Assefa et al. (1999) demonstrated that activation of p38 protected HeLa cells from apoptosis after photodynamic therapy with hypericin, suggesting that activation of p38 might afford some cytoprotective effect in non-neuronal cells. Activation of ERK leads to phosphorylation of several downstream effectors, including protein kinases and transcription factors responsible for regulating genes that enhance cell proliferation and protect from apoptosis (Lewis et al., 1998). Interestingly, there is one biochemical link between ERK and Bcl-2 family namely the regulation of the phosphorylation of Bad. ERK can phosphorylate Bad at Ser112 and then inactivate Bad by sequestering it in the cytosol and preventing its dimerization with Bcl-XL (Rice et al., 2003). Furthermore, ERK activation has been associated with inhibition of apoptosis by enhanced XIAP transcription in acute myeloid leukemia cells and THP-1 cells (Milella et al., 2001). As we know, JNK, p38 and ERK can only exert their activity after phosphorylation, and the upregulation or the downregulation of these phosphorylated proteins indicates the activity of the corresponding proteins. Since MAPKs play a significant role in regulating the apoptosis of cancer cells, the protein level of MAPKs was investigated. In this study, Q39 obviously increases phosphorylation of JNK and decreases in phosphorylations of ERK and p38. In contrast, there is no change in the expression of ERK. These observations suggest that the activity of MAPK family may be involved in the Q39-mediated apoptotic process.

Moreover, there are several lines of evidence to indicate that high level of HIF-1α, VEGF and their resultant activity promote tumor progression and the development of treatment resistance (Sohda et al., 2004; Koukourakis et al., 2002; Ryan et al., 2000). We correlated the effects of Q39 on HIF-1α and VEGF expression in response to hypoxia, and found that Q39 blocks the induction of HIF-1α and VEGF in hypoxia. However, further studies are required to clarify the reason why HIF-1α level accidentally increases at 12 h (repeat 3 times). In many cancers, the HIF-1 pathway is not only activated by low oxygen tension, it is also induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways. Increased HIF-1α protein synthesis via the activation of PI3K-Akt-mTOR or MAPK pathways is a common theme accounting for the upregulation (Ke and Costa, 2006; Zhang et al., 2006). Thus, the previous studies are warranted to determine whether inhibition of these phosphorylation events by Q39 are related to decreased HIF-1α synthesis. Here, we investigate the protein relative to the MAPK pathways, and show that Q39 significantly decreases p-ERK and increases p-JNK, indicating that Q39 may influence MAPK family to block HIF-1α protein synthesis, and finally decrease HIF-1α level.

Taken together, the present studies indicate that Q39-induced apoptosis is mediated by mitochondrial permeability changes that lead to caspase-dependent cytotoxicity. Our studies also show that Q39 decreased HIF-1α protein levels under hypoxic conditions, which is associated with a decrease in HIF-1α protein synthesis. Precisely how Q39 inhibits HIF-1α protein synthesis remains to be elucidated. A better understanding of these mechanisms will undoubtedly provide new insights into HIF biology and opportunities for pharmacologic and therapeutic intervention in HIF-1-driven tumor growth.

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