XQ2, a Novel TPZ Derivative, Induced G2/M Phase Arrest and Apoptosis under Hypoxia in Non-Small Cell Lung Cancer Cells

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Hypoxia is one of the inevitable circumstances of various tumors. It controls various levels of regulation in tumor progression and results in tumor resistance to radiotherapy and chemotherapy. Here we investigated a synthetic TPZ derivative, N-ethoxymethyl-3-aminomethyl-1,2,4-benzotriazine-1,4-dioxide (XQ2), a novel compound that induced anti-cancer effects both in normoxia and in hypoxia, cell proliferation assay found that XQ2 exhibited a potent inhibitory effect on the tested cancer cell lines both in normoxia and in hypoxia. Flow cytometry and western blot studies indicated that XQ2 induces G2/M arrest and a caspase-dependent apoptosis in A549 cells. Additionally, intracellular reactive oxygen species (ROS) appear to play a key role in the anticancer effect of XQ2 in hypoxia. Taken together, our data suggest that XQ2 exerted anticancer action by suppressing the ROS level and triggering cell-cycle arrest and the caspase-dependent pathway, which is associated with apoptosis.

Key words: caspase-dependent apoptosis; hypoxia; NSCLC; ROS; XQ2

Lung cancers, which are classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma), is the leading cause of male and female cancer death worldwide. Hypoxia is associated with a worse prognosis in patients with NSCLC undergoing tumor resection and leads to less sensitivity to chemotherapeutic agents.1) Thus it is crucially important to develop better therapeutic strategies for the management of NSCLC in hypoxia.

Hypoxia contributes to tumor progression and limits the response of tumors to radiotherapy and chemotherapy.2) However, tumor hypoxia can be turned to advantage by exploiting it to activate bioreductive prodrugs. So far, the most advanced of the hypoxia-selective bioreductive drugs in clinical evaluation is TPZ (Tirapazamine; 1,2,4-benzotriazin-3-amine 1,4-dioxide, SR 4233), which showed potent activity in combination with cisplatin in a Phase III trial against non-small cell lung cancer.3) TPZ is an anticancer drug that targets topoisomerase II. It is preferentially active under hypoxic conditions. The drug itself is not harmful to cells; rather, it is reduced to a toxic radical species by an NADPH cytochrome P450 oxidoreductase. It has been well determined that hypoxia-selective compound induced apoptosis, including TPZ, is mediated by mitochondrial permeability changes in hypoxia.4,5)

Previous studies have found that the essential executors of apoptosis are the caspases, a family of conserved cysteine aspartate-specific proteases,6) that are activated via proteolytic cleavage. Generally, two major pathways for apoptosis have been described.7) In the extrinsic (or death receptor) pathway, apoptosis is mediated by death receptors (such as Fas or tumor necrosis factor receptors)8) and involves caspase-8 activation, while in the intrinsic (or mitochondrial) pathway, diverse proapoptotic signals stimulate the translocation of cytochrome c from mitochondria to cytoplasm which promotes caspase-9 activation.

Reactive oxygen species (ROS), such as superoxide and peroxide, have been implicated as oxygen sensors, and thus linked to the regulation of HIF-1 under limited oxygen supply.9) In this regard, there is acute controversies in the literature as to whether ROS levels increase or decrease under hypoxic conditions.10–12) Moreover, ROS appear to have dual effects on HIF-1: a number of reports highlighted the role of ROS in HIF-1α induction,13,14) whereas ROS have also been found to be able to destabilize HIF-1α under hypoxic and non-hypoxic conditions.15) Consequently, the regulatory mechanisms by which HIF-1 is regulated by ROS under hypoxic condition are far from fully elucidated.

Here, we report that XQ2, a novel TPZ derivative, induces G2/M arrest and apoptosis in human lung adenocarcinoma via a process that involves the Bax/Bcl-2-dependent mitochondrial signaling pathway. Our results also indicate that XQ2 decreased the expression of hypoxia-induced HIF-1α protein via the ROS-dependent pathway.

Materials and Methods

Drugs and chemicals. XQ2 was supplied by Professor of Yongzhou Hu. A stock solution of XQ2 (100 μM) was prepared with dimethyl sulfoxide (DMSO) and was stored at −20 °C for in vitro testing. The

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stock solution was further diluted with an appropriate assay medium immediately before use. A general caspase inhibitor, z-VAD-fmk (R&D Systems, Minneapolis, MN), was reconstituted in DMSO. The final DMSO concentration did not exceed 0.1% throughout the study. Antibodies for HIF-1α, procaspase-9, procaspase-3, poly (ADP-ribose) polymerase (PARP), Bax, β-actin, and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Bcl-2, cleaved caspase-9, and caspase-3 were from Cell Signaling Technology, (Beverly, MA).

Cell culture and establishment of hypoxia culture condition. Five human cancer cell lines were employed in this study. Androgen independent prostate carcinoma PC3, chronic myeloid leukemia K562, hepatocellular carcinoma SMMC-7721, human epidermoid KB carcinoma, and human lung adenocarcinoma A549 were purchased from the Cell Bank of the China Science Academy (Shanghai, China). These cells were maintained in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) plus 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml), and were incubated at 37°C in a humidified atmosphere with 5% CO2. 

Estimation of intracellular reactive oxygen species (ROS). Intracellular ROS were detected by flow cytometry using 2,7'-dichlorodihydrofluorescein diacetate (H2-DCFDA, Molecular Probes, Leiden, Netherlands). A549 cells were treated with and without XQ2 for 0.5–3 h, and then H2-DCFDA (15.0 μm) was added and the mixture was incubated for 30 min. The cells were then washed and re-suspended in 1 ml PBS. Flow cytometric analysis was carried out using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). In each study, 10,000 cells were counted. The fluorescence of DCF was detected at excitation and emission wavelengths of 488 and 530 nm respectively. 10

Western blotting. Protein samples from the A549 cells were prepared as described previously. Protein extracts were resolved by 8%–15% SDS-PAGE and electrobotted onto PVDF membranes, and then western blot analysis was carried out using specific primary antibodies against procaspase-9, procaspase-3, cleaved caspase-9, cleaved caspase-3, PARP, Bax, Bcl-2, and HIF-1α (1:500) by incubation with a horseradish peroxidase-conjugated secondary antibody (1:5,000). The signals were visualized by the enhanced chemiluminescence detection system (Biological Industries, Beit Haemek, Israel). As a loading control, the blots were probed with a specific antibody against human α-tubulin (1:1,000).

Statistical analysis. Data are presented as mean ± SD for three separate experiments. Comparisons between groups were performed by one-way ANOVA, followed by Bonferroni post hoc tests for multiple comparisons, with p < 0.05 considered significant.

Results

XQ2 inhibited human cancer cell proliferation both in normoxia and in hypoxia

In the present study, we examined the effects of XQ2 (Fig. 1A) on the growth of five human tumor cell lines, and found that it displayed strong anti-proliferative properties both in hypoxia and in normoxia over 72 h of treatment (Fig. 1B). A549 and SMMC-7721 cells, in particular, were the most sensitive to XQ2 in hypoxia, while KB cells were resistant. We further determined the effects of TPZ on A549, and observed that XQ2 and TPZ showed similar hypoxic selectivity in A549 cells. Selective action (IC50 normoxia/IC50 hypoxia) 3-fold of XQ2 and 4-fold of TPZ was observed (Fig. 1B). Since TPZ is usually used to target non-small cell lung cancer (NSCLC), including A549, the subsequent studies were performed on this cell line.

XQ2 induced G2-M arrest and apoptosis of A549 cells in hypoxia

In order to determine the mechanism involved in XQ2-induced growth retardation, apoptotic cells were evaluated by flow cytometry (Fig. 2A–C). A concentration-dependent number of apoptotic cells was detected, and maximum levels of apoptotic cells were detected at 40 μm in response to XQ2 (30.39 ± 1.4%), which represents an 8.2-fold increase with respect to control (3.72 ± 0.51%) (Fig. 2B, C). However, XQ2 (20 μm) did not obviously induce apoptosis during treatment for 48 h in normoxia (Fig. 2A). Similar results were obtained with the positive control TPZ under both normoxia and hypoxia, and a more competitive potential of XQ2 was apparent when they were taken together.

To determine whether XQ2-induced anti-proliferation involves alterations in cell-cycle progression, flow cytometric analysis was included in this study. As shown in Table 1 and Fig. 2D, E, all of the indicated concentrations of XQ2 (10, 20 and 40 μm) caused significant G2/M arrest following 48 h of treatment in hypoxia (F(4, 10) = 1.683, p < 0.001). Cell-cycle phase distribution was especially sensitive to XQ2, since this hypoxic cytotoxin induced the highest ratio in G2/M arrest, in a dose-dependent manner (17.3%, 40.4%, and 26.4% vs. 6.1% in the control cells). XQ2 (40 μm), the highest concentration in this study, also results in G2/M arrest in normoxia (F(4, 10) = 2.735, p < 0.001). In addition, treatment with XQ2 (20 and 40 μm) also stimulated accumulation of the cells in the S phase (24.4% and 44.1% vs. 16.2% in the control cells) in hypoxia (F(4, 10) = 2.158, p < 0.001), but did not affect the S phase in normoxia. These data indicate that XQ2-induced apoptosis and G2/M arrest made a contribution to the inhibition of cell proliferation in hypoxia.

Effects of XQ2 on Bcl-2 family, procaspases-9 and -3 cleavage, and PARP processing

To determine whether XQ2-triggered apoptosis has to do with the activation of caspases, A549 cells were pre-treated with a broad-spectrum caspase inhibitor, z-VAD-fmk. Apoptosis caused by XQ2 was subtotally suppressed in the presence of the inhibitor (Fig. 3A), which suggests that XQ2 had anti-proliferative action
Fig. 1. XQ2 Inhibited Human Cancer Cell Proliferation under Hypoxic Condition.
A, Chemical structures of synthetic MBL. B, A549 (a), KB (b), PC3 (c), SMMC-7721 (d), or K562 (e) cells were plated in 96-well plates (5 × 10^3 per well) for 24 h and subsequently treated with various concentrations of XQ2 (0–50 μM) and TPZ (0–50 μM) for 72 h in 20% O_2 and 0.6% O_2, and cytotoxicity was analyzed with MTT method. The points represent the mean fractional survival; error bars represent standard deviation. Values represent the means ± SD for three separate experiments performed in triplicate.

Fig. 2. XQ2 Induces G2–M Arrest and Apoptosis of A549 Cells in Hypoxia.
A and B, A549 cells were treated with DMSO, 10, 20, or 40 μM XQ2 for 48 h in 20% O_2 (A) and 0.6% O_2 (B), respectively. After treatment, apoptosis was assessed by propidium iodide (PI) staining of the lysed cell nuclei as described in Materials and methods. The DNA content of 15,000 events was analyzed by flow cytometry. C, quantitative analysis of results from A and B. Columns, mean percentage of apoptotic cells from three replicate experiments; error bars represent standard deviation. D and E, A549 cells were incubated as above, and cell-cycle distribution was analyzed by flow cytometry.
in hypoxia through a caspase-dependent mechanism. Moreover, immunoblotting studies suggested that apoptosis induction occurs via the intrinsic pathway, since XQ2 stimulated cleavage of inactive pro-caspase-9 to the active 35–37 kDa fragments (Fig. 3B), but did not significantly promote pro-caspase-8 hydrolysis (data not shown). Since caspase-3 is the main effector caspase in apoptosis, we also analyzed to determine whether this hypoxic cytotoxin induces hydrolysis of zymogen by immunoblot analysis. Cleavage of pro-caspase-3 to 17–19 kDa fragments significantly increased in XQ2-treated cells under hypoxia (Fig. 3B). Poly (ADPribose) polymerase protein (PARP), which is normally involved in DNA repair and is known to be a substrate for caspase-3, in our result was effectively hydrolyzed to the 85-kDa fragment following XQ2 (40 μM) treatment in hypoxia. However, caspase-3 and PARP were not activated by XQ2 in normoxia. These observations indicated that activation of caspase cascade might play a crucial role in XQ2-induce apoptotic death in A549 cells in hypoxia.

It is often assumed that Bcl-2 family molecules regulate the release of cytochrome c from the mitochondria to the cytosol, which triggers cell apoptosis,18 Bcl-2 has been found to localize to the mitochondrial membrane and to stabilize mitochondrial functions, thereby suppressing the release of pro-apoptotic effector molecules. Conversely, translocation to the mitochondria of Bax induces cytochrome c release from the mitochondria.19 As shown in Fig. 3C and D, exposure of A549 cells to XQ2 caused a significant decrease in Bcl-2 and a robust increase in Bax protein levels after 48 h of treatment in hypoxia, but, the expression level of Bcl-2 protein was not affected by XQ2 in normoxia. The results suggest that XQ2-mediated alteration of Bcl-2 family protein expression is a possible mechanism of the XQ2-mediated release of cytochrome c in hypoxia.
XQ2 decreased HIF-1α protein expression through inhibition of ROS generation in hypoxia

To investigate the role of ROS in the regulation of HIF-1α protein expression, A549 cells were exposed to hypoxia for the indicated time periods. ROS production reached maximum after a 3-h exposure hypoxia (Fig. 4A) as well as maximum induction of HIF-1α protein expression (Fig. 4C, lane 5). In an attempt to understand the effect of XQ2 on HIF-1α expression, we detected HIF-1α protein expression by western blotting. It decreased considerably under the indicated treatment of XQ2 in hypoxia. To further clarify whether ROS production mediated the marked decrease in HIF-1α protein accumulation, 10 μM XQ2 was employed in the test. It was found that XQ2 could suppress ROS levels obviously in hypoxia (Fig. 4B). Compared with the normoxic condition, a similar ROS level was detected when the XQ2 present reached 40 μM. We also examined ROS generation under treatment with XQ2 in the presence and the absence of a free radical scavenger, N-acetyl-L-cysteine (NAC). In normoxia, XQ2 and NAC treatment slightly changed the ROS level. However, NAC potently inhibited ROS generation, with a 2.1-fold decrease with respect to control (Fig. 4D). Moreover, ROS level was significantly decreased by treatment with XQ2 (20 μM) alone. In the presence of NAC, ROS production was not obviously altered as compared to XQ2 alone, suggesting that XQ2 is a free-radical scavenger without synergism between NAC, and further destabilizes HIF-1α protein accumulation via suppression of ROS generation.

Discussion

The poor prognostic outcome for lung cancer is due to its resistance to current therapies. Hypoxia adaptation is a critical factor determining the malignancy of lung tumors, and the activation of a series of hypoxia-induced genes is a crucial event supporting the hypoxic adaptation of lung tumors.20) Successful treatment with chemotherapeutic agents is largely dependent on their ability to trigger cell death in tumor cells in hypoxia; therefore, novel hypoxia-inducers of apoptosis should provide a new therapeutic approach in anti-cancer design. Several previous studies indicate that TPZ and its derivatives exert anti-tumor activity by inducing apoptosis in cancer cells.21) Besides, TPZ combined with several anti-cancer drugs has been well studied in phase II trails on advanced non small cell lung cancer (NSCLC), which further confirms that TPZ and its derivatives are the exploitable therapeutic agents in NSCLC.

In this study, we synthesized a series of compounds containing a 3-amino-1,2,4-benzotriazine-1,4-dioxide group (data not shown), and evaluated their potential anti-proliferative properties using a wide number of tumor cell lines both in hypoxia and in normoxia. We found that N-ethoxymethyl-3-amino-1,2,4-benzotriazine-1,4-dioxide (XQ2) displayed its anti-proliferative properties in a cell type-specific manner in hypoxia. A549 and SMMC-7721 cells were highly sensitive to XQ2 cytotoxicity in hypoxia while, at the other extreme, KB cells were almost completely resistant. We speculate
that 3-ethoxymethylamino of the XQ2 chemical structure contribute to its anti-oxidative activity.

Since many anti-cancer agents arrest the cell cycle at the G1, S or G2/M phase and then induce apoptotic cells,22,23 we observed to determine whether the decrease in cell proliferation due to XQ2 was due to cell cycle arrest and apoptosis. We examined the cell cycle distribution in A549 cells treated with XQ2, and found that inhibition of cell viability was caused by significant cell-cycle arrest at the G2/M phase in hypoxia, accompanied by an increase in sub-G1 fraction, indicating apoptotic cell death. XQ2 (20 and 40 μm) also induced accumulation of cells in the S phase, which suggest that XQ2 had pleiotropic effects in exerting anti-tumor ability in hypoxia. Similar results have been obtained with the novel sulfonamide anticancer agent E7070 in A549 cells.24 We also found that XQ2 (40 μm) displayed effects on cell-cycle progression, whereas it did not induce the apoptosis of A549 cells in normoxia, which implies that the concentration of XQ2 (40 μm) induced cell growth inhibition via cell cycle arrest during normoxic conditions. Thus, additional studies are required to ascertain whether XQ2 can induce the apoptosis of A549 cells in normoxia.

Under hypoxia activates genes that are essential for cellular adaptation to changes in oxygen levels. Hypoxic gene regulation is mediated mainly through a ubiquitous transcription factor, hypoxia-inducible factor-1 (HIF-1).25 HIF-1 regulates gene expression, like apoptosis-related or cell cycle regulators, by binding the hypoxia response element (HRE) found on the promoters of target genes. This is critical to chemotherapy resistance in hypoxia. Accumulating evidence indicates that mitochondria play a pivotal role in the apoptotic process in mammalian cells. Disruption of the mitochondrial membrane potential (∆Ψm) is considered to be an indicator of mitochondria dysfunction of and generally is defined as an early stage of apoptosis, preceding the efflux of small molecules from the mitochondria (including cytochrome c, apoptosis-inducing factor, etc.) and to be followed by caspase-9/caspase-3 cascade activation.26 Recent reports indicate that hypoxic cytotoxin-induced apoptosis operates through a mitochondrial-dependent activation of caspase-3 and -9 in human chronic myeloid leukemia K562 cancer cells,27 and in human neuroblastoma SMS-SAN, SK-N-BE, and CHLA-15 cells.28 In the present study, we found that marked activation of caspases (caspase-3 and -9) by XQ2 in hypoxia and treatment of A549 cells with the pan broad-spectrum caspase inhibitor z-VAD-fmk drastically blocked the apoptosis induced by XQ2 in hypoxia, and we observed marked activation of caspases (caspase-3 and -9) by XQ2. These results suggest that intrinsic mitochondria-mediated caspase activation signaling pathway plays a crucial role in XQ2-triggered apoptosis.

The Bcl-2 family of proteins is regarded as key regulators of apoptosis induced by hypoxia in a HIF-1-dependent manner. HIF-1α can regulate Bax and Bcl2 activation. Bcl-2 is considered to be antiapoptotic, whereas Bax is regarded as pro-apoptotic. Therefore, the balance between pro-apoptotic Bax and antiapoptotic Bcl-2 can be associated with apoptosis. Bax and Bcl-2 are responsible for either the induction or the prevention of mitochondrial membrane permeability, which is important in regulating the release of cytochrome c from the mitochondria into the cytosol, leading to activation of the caspase cascade as with caspase-9 and caspase-3 and induction of apoptotic cell death.27 In this study, XQ2-induced apoptosis in A549 cells was accompanied by up-regulation of Bax and downregulation of Bel-2. The Bax/Bcl-2 ratio induced by 10–40 μm XQ2 ranged from 14.9-fold to 156.7-fold in hypoxia, normalized against the Bax/Bcl-2 level without XQ2 in normoxia. We found that XQ2 triggered the overexpression and integration of Bax into mitochondrial membrane and downregulation and efflux of Bel-2, and that this was responsible for the commitment of the cells to apoptosis.

Previous studies indicate that HIF-1α can be modulated by reactive oxygen species (ROS), whereas there is controversy as to whether an increase or a decrease in ROS production is required for hypoxic signaling. There are reports indicating an increase in HIF-1α protein by scavenging endogenous ROS, e.g., the hydroxyl radical at perinuclear endoplasmic reticulum pockets, using dihydrotrihydromannine (DHR) in HepG2 cells.29 In contrast, other reports indicate for A549 cells, by reference to an increase in the fluorescence of H2DCFDA under hypoxia, that increasing amounts of ROS provoke HIF-1α accumulation.30 Our experiments supported the latter finding that ROS increases account for HIF-1α stabilization and activation under hypoxic conditions.

In conclusion, XQ2, evaluated in the present study, was strongly cytotoxic to human lung adenocarcinoma cell lines, involving cell-cycle perturbation and caspase-dependent apoptosis in hypoxia. XQ2 also inhibited hypoxia-induced HIF-1α protein accumulation expression in A549 cells by suppressing ROS production. Chemical synthesis of this kind of compound might allow the discovery of new and highly specific antitumor agents against hypoxic cells.

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