Original Paper

The Cytotoxic Effect of the Benzene Metabolite Hydroquinone is Mediated by the Modulation of MDR1 Expression via the NF- κ B Signaling Pathway

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Key Words

Hydroquinone • MDR1 • NF- κ B

Abstract:

Background/Aims: Benzene is a toxic chemical whose leukemogenic effects have been studied for decades. The mechanisms of benzene-induced toxicity and leukemogenicity are not fully understood, although the involvement of several pathways has been suggested, including oxidative stress, DNA damage, cell cycle regulation and programmed cell death. In the present study, we investigated the effect of hydroquinone (HQ), a major benzene metabolite, on the viability of bone marrow derived mesenchymal stem cells (BMSCs) and explored the underlying mechanisms. **Methods:** First, we study the the effect of HQ on BMSCs cell viability, apoptosis and the expressions of MDR1 and NF- κ B. Then we investigate the MDR1 on cell viability and cell apoptosis for BMSCs under HQ treatment. Finally, we studied the impact of nuclear factor κ B (NF- κ B) on the expression of MDR1. **Results:** Our results showed that HQ decreased cell viability and promoted cell apoptosis of BMSCs, as determined by the MTT assay and flow cytometry. Western blotting and quantitative PCR showed that HQ downregulated the expression of the *MDR1* gene by inhibiting the activation and nuclear translocation of the transcription factor NF- κ B. Overexpression of MDR1 attenuated the inhibitory effect of HQ on cell viability in BMSC. **Conclusion:** The results of the present study suggest the involvement of the multidrug resistance membrane transporter MDR1 and the NF- κ B pathway in the cytotoxicity of benzene and its metabolites. Further studies are necessary to clarify the role of the pathways involved and the crosstalk between them in mediating the effects of HQ in bone marrow progenitor cells.

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Introduction

Benzene is a clear, colorless liquid that volatilizes in air and is commonly present in the atmosphere as a result of emissions from burning coal and oil, gasoline vapors, motor vehicle exhaust, cigarette smoke and wood-burning among other sources [1]. Human exposure to benzene occurs through inhalation, dermal absorption, the ingestion of contaminated food and drinking water, and active and passive tobacco smoking [2, 3]. Benzene is converted into toxic metabolites including benzene oxide, phenol, hydroquinone (HQ), catechol, and 1,2,4-benzenetriol in the liver [4]. Benzene exposure has been shown to be correlated with the incidence of leukemia, in particular acute myeloid leukemia, but also acute and chronic lymphocytic leukemia, non-Hodgkin's lymphoma, multiple myeloma, and aplastic anemia [5, 6]. Exposure to low levels of benzene has been associated with increased risk of myelodysplastic syndrome and decreased resistance to infection [7]. HQ, the most abundant pro-oxidant compound in cigarette smoke, can bind both DNA and protein and can induce oxidative stress. It can travel to the bone marrow, where it is oxidized to highly toxic quinones, thus contributing to the toxicity of benzene [8-10]. Despite extensive research into the effects of benzene and its metabolites, the mechanism underlying the toxicity of benzene remains to be fully elucidated.

The transcription factor nuclear factor κ B (NF- κ B) inhibits apoptosis and induces resistance to chemotherapeutic drugs in cancer cells [11]. In unstimulated cells, NF- κ B is retained in the cytoplasm by the inhibitor I κ B, which is phosphorylated by the IKK kinase complex in response to different stimuli, promoting its ubiquitination and degradation, and freeing NF- κ B to translocate to the nucleus and induce the transcription of its target genes. The involvement of NF- κ B in drug resistance through the transactivation of the *MDR1* gene has been proposed previously, and an NF- κ B binding site was identified in the human *MDR1* gene. Drug resistance can be mediated by decreased cellular drug uptake, which can be associated with overexpression of P-glycoprotein (P-gp), a membrane transporter that is encoded by the *MDR1* gene. The transcriptional regulation of MDR1 has been studied extensively and a number of transcription factors have been implicated in the modulation of its expression including Ras, Sp1, p53, NF- κ B, and PKC [12-16]. In addition, extracellular stimuli such as heat shock and chemotherapeutic agents, as well as DNA methylation and histone acetylation have been involved in the regulation of MDR1 expression [17-20].

Mesenchymal stem cells (MSCs) are a multipotent population of non-hematopoietic cells that can differentiate into bone, cartilage, adipose and fibrous tissues *in vitro* or *in vivo* [21, 22]. The development and optimization of techniques for the isolation, purification and expansion of bone marrow derived MSCs (BMSCs) stimulated extensive research into their characterization and differentiation potential. Furthermore, the finding that BMSCs secrete specific growth factors and cytokines that direct each differentiation pathway and that modulation of these molecules can induce a specific phenotypic lineage suggested the potential of these cells for tissue repair [23]. The multi-lineage potential of MSCs and their relative ease of handling *in vitro* have made them a powerful tool for tissue engineering [24, 25].

In the present study, we examined the effect of the benzene metabolite HQ on the expression of MDR1 in BMSCs and explored the mechanisms underlying benzene toxicity to identify novel targets for the treatment of chronic benzene poisoning.

Materials and Methods

Isolation and culture of BMSCs [26]

Newborn rabbits were sacrificed by sodium pentobarbital overdose under sterile conditions. The bilateral femurs were dissected with the proximal and distal ends snipped off, and bone marrow tissue was flushed out using a 1-mL sterile syringe with minimum essential medium (MEM; Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal calf serum and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin). Following centrifugation at 800 rpm for 5 min, the cell pellets were collected and

resuspended in fresh culture medium. Cells (1×10^5) were cultured in a 25-cm² culture flask at 37°C and 5% CO₂. The culture medium was changed every three days. Cells in the third passage were harvested for subsequent experiments.

Cell viability assessment

Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells at a density of 1×10^6 cells/mL were treated as indicated and then incubated with MTT (5 mg/mL in PBS) to a final concentration of 0.2–0.5 mg/mL for 4 h. After removing the supernatant, the formazan precipitate was dissolved in DMSO and absorbance was read in an ELISA plate reader at 570 nm. The results were expressed as a percent of the untreated control and all experiments were repeated three times.

Flow cytometric analysis of apoptosis with the Annexin V-FITC/PI assay

Cells were treated with HQ (0, 25, 50, 75, 100 and 125 μ M) for 24 h prior to apoptosis assessment, which was performed as described previously (Siew et al., 2012). Briefly, 1×10^6 cells were collected and resuspended in 150 mL Annexin V buffer containing 2.5 ml FITC-conjugated Annexin V and incubated for 15 min in the dark. Propidium iodide (10 mL of 50 mg/mL stock in PBS) was then added and samples were subjected to flow cytometric analysis using FACS CANTO II (BD Bioscience).

qt-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Shanghai, People's Republic of China), according to the manufacturer's protocol. One microgram of total RNA was used to prepare cDNA by reverse transcription using a PrimeScript RT reagent kit (Takara, Dalian, People's Republic of China). The primer sequences were as follows (Table 1). cDNA was amplified using a SYBR Premix Ex Taq kit (Takara, Dalian, People's Republic of China) and an Mx3000p instrument (Agilent). The PCR protocol was as follows: one cycle of denaturation at 95°C for 30 s; 40 cycles of denaturation at 95°C for 5 s; and annealing at 60°C for 20 s. PCR products were analyzed using the $\Delta\Delta$ CT method with β -actin as the standard gene.

Western blot analysis

For preparation of nuclear extracts, cells were harvested, rinsed, and lysed in a hypotonic buffer consisting of 10 mM HEPES (pH 7.4), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride), protease inhibitors (0.5 mM phenylmethyl sulfonyl fluoride, 1 μ g/mL pepstatin A, 2 μ g/mL leupeptin, and 2 μ g/mL aprotinin), and 1% Triton X-100.

For total cellular protein extracts, a total protein extraction kit (KeyGen Biotech, Nanjing, People's Republic of China) was used. For western blot analysis, lysates were separated on 15% polyacrylamide sodium dodecyl sulfate gels and electroblotted onto Immobilon-P membranes (Millipore Corp., Bedford, MA). After blocking in 5% fat-free powdered milk in Tris-buffered saline with 0.05% Tween 20, membranes were incubated with primary antibodies against MDR1 (1:500; Abcam), β -actin (1:500; Santa Cruz Biotechnology, Inc.), NF- κ B p65 (1:1000; Santa Cruz Biotechnology, Inc.) for overnight at 4°C. Secondary antibodies against horseradish peroxidase-conjugated anti-mouse IgG were purchased from Cell Signaling Technology (Beverly, MA, USA). Proteins were visualized with enhanced chemiluminescence.

Immunofluorescence

Cells were grown on coverslips and cultured for 24 h. After treatment with the indicated doses of HQ, the cells were fixed in 4% paraformaldehyde for 15 min, washed in PBS, and treated for 15 min with PBS containing 0.1% Triton X-100. The cells were then washed, blocked with 10% bovine serum albumin (BSA) for 1 h at room temperature, and incubated at 4°C overnight with NF- κ B p65 antibody (1:100) diluted in 0.1% BSA. After extensive washing, a 1:200 dilution of Dylight 649 conjugated goat anti-mouse immunoglobulin was applied as the

Table 1. The primer sequences used for quantitative real-time PCR

Primer	Sequence(5'-3')
MDR1-F	F:GTGAGGTATCTGCGGGAAATTA
MDR1-R	R: GTGACATCTTCTCGGCCATAG
GAPDH	F:GGCAAGTTCAACGGCACAG
GAPDH	R:CGCCAGTAGACTCCACGAC

secondary antibody for 1 h at room temperature. Nuclear staining was achieved by incubating cells in DAPI for 5 min. The slides were then washed and photographed with an OLYMPUS 1X71 fluorescence microscope.

Transient transfections and luciferase assays

For DNA transfection, cells were plated at a density of 2.0×10^5 cells per well in six-well culture dishes. After 24 h, the cells were transfected with a construct containing a luciferase reporter gene driven by a fragment of the *MDR1* promoter region together with expression vectors for the NF- κ B protein. The p225 plasmid contains a 976 bp PCR-generated fragment of the *MDR1* gene that was subcloned into the pGL3-Basic vector (Promega, Madison, WI, USA) upstream of the luciferase reporter gene. Cells were transfected using the Fugenet 6 transfection reagent as recommended by the manufacturer (Roche, Mannheim, Germany). A Renilla luciferase plasmid was also cotransfected in each experiment as an internal control for transfection efficiency. Approximately 24 h after the beginning of the transfection, cells were lysed and Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Results were expressed as luciferase activity relative to the empty vector control.

Statistical analysis

Data were presented by as means $\pm$ standard deviations (SD). Statistical analysis was performed by using the statistical package social science (SPSS) program, version 17.0. Student T-test was used to determine statistical significance. A p-value less than 0.05 was considered significant and p-value less than 0.01 was considered highly significant.

Results

HQ affects the viability and apoptosis of BMSCs

BMSCs were incubated with increasing doses of HQ (Fig. 1A) or with a single dose of HQ (125 μ M) for different times from 2 to 10 h (Fig. 1B) and cell viability was assessed using the MTT assay. HQ caused a significant dose and time dependent inhibition of BMSC viability ($p < 0.05$). Assessment of apoptosis in response to increasing concentrations of HQ from 25 μ M to 125 μ M showed a dose dependent significant increase in the rate of BMSC apoptosis (Fig. 1C, D).

HQ downregulates the expression of MDR1 mRNA and protein

BMSCs were exposed to increasing concentrations of HQ from 25 μ M to 125 μ M and the expression of MDR1 was assessed by quantitative PCR (qPCR) (Fig. 2A) and western blotting (Fig. 2B). The results showed that HQ downregulated the expression of MDR1 in a dose-dependent manner at the mRNA and protein levels.

Effect of MDR1 overexpression or silencing on the response of BMSCs to HQ

BMSCs were transfected with vectors overexpressing or silencing MDR1, and cell viability and apoptosis were assessed in response to treatment with increasing concentrations of HQ. Ectopic expression of MDR1 attenuated the effect of HQ on the inhibition of cell viability (Fig. 3A) and the induction of apoptosis (Fig. 3B), and the effect was stronger in response to HQ at 75 and 100 μ M ($p < 0.05$). Knockdown of MDR1 expression, on the other hand, enhanced the effect of HQ on cell viability and apoptosis, but the effect was only detectable at certain concentrations.

HQ inhibits the nuclear translocation of NF- κ B

To determine whether the effect of HQ on BMSC viability via the modulation of MDR1 expression was mediated by the NF- κ B pathway, the expression of the nuclear NF- κ B p-p65 was assessed in nuclear extracts of BMSCs treated with different concentrations of HQ. The results showed that the levels of the nuclear NF- κ B p-p65 decreased in response to HQ in a dose-dependent manner (Fig. 4A and B). Furthermore, immunofluorescence detection of NF- κ B p65 in BMSCs treated with different concentrations of HQ showed a dose-dependent

Fig. 1. Cytotoxicity of HQ in BMSCs. (A) BMSCs were exposed to HQ (25, 50, 75, 100, 125, and 150 μM) for 24 h or (B) 125 μM HQ for 2, 4, 6, 8, and 10 h and cell viability was assessed using the MTT assay. (C and D) BMSCs were exposed to HQ (25, 50, 75, 100, and 125 μM) for 24 h and apoptosis was determined by Annexin V-FITC/PI flow cytometry. All assays were repeated three times. Results are shown as the mean ± SD of three independent experiments (* P<0.05, ** p<0.01).

Fig. 2. Effect of HQ on the expression of MDR1. (A) qRT-PCR analysis of MDR1 mRNA levels and (B) Western blot analysis of MDR1 protein levels in cells treated with different doses of HQ (25, 50, 75, 100, and 125 μM) for 24 h (* P<0.05, ** p<0.01).

inhibition of the nuclear localization of the p-p65 subunit, as observed in merged images with the nuclear stain DAPI (Fig. 4C). Taken together, these results suggested that HQ inhibits the activation of NF-κB in BMSCs.

Fig. 3. Effect of MDR1 overexpression or knockdown on BMSC viability in response to HQ. (A and B) BMSCs were transfected with vectors overexpressing(OE) or silencing MDR1(KO) and cell viability (A) and apoptosis (B) were assessed in response to treatment with different doses of HQ (25 – 125 μ M) for 24 h(* P <0.05).

Fig. 4. Effect of HQ on NF- κ B activation in BMSCs. (A) The expression of p65 was assessed by Western blotting in nuclear extracts of BMSCs treated with different concentrations of HQ (* P <0.05, ** p <0.01). (B) Results of densitometric scan of the blot shown in (A). (C) Immunofluorescence detection of NF- κ B p65 (DyLight 649, Dy) and nuclear staining with DAPI and merged images (original magnification $\times$ 400).

Inhibition of the NF- κ B transduction pathway reduces induced MDR1 expression

Incubation of BMSCs with NF- κ B inhibitor PDTC blocked the activates NF- κ B Signaling Pathway. There was no significant inhibition in cells pretreated with PDTC at 10 μ M. Cells pretreated with PDTC at 25 and 50 μ M for 1h showed complete inhibition of the NF- κ B signal pathway as show in Fig 5A. Reduced MDR1 mRNA expression and protein was detected in BMSCs pretreated with inhibitor PDTC using WB and RT-PCR. This trend showed relationship to PDTC pretreatment, as shown in Fig 5A, B.

Fig. 5. Inhibit NF- κ B downregulate the expression of MDR1. (A) Cells were treated with different doses of an NF- κ B inhibitor PDTC and the expression of NF- κ B p65 or MDR1 were assessed by Western blotting. The β -actin (for total p65,MDR1 samples) and Histone H3 (for nuclear-p65 samples) were respectively used as a control for sample loading. (B) BMSCs were treated with different doses of an NF- κ B inhibitor PDTC and the mRNA of MDR1 was assessed by qRT-PCR.

Fig. 6. Effect of NF- κ B on the expression of MDR1. (A) Cells were transfected with different doses of an NF- κ B p65 expression plasmid and the expression of NF- κ B p65 or MDR1 were assessed by western blotting. (B) BMSCs were transfected with a MDR1 promoter-luciferase reporter plasmid and the effect of NF- κ B p65 was assessed by dual-luciferase reporter assay.

NF- κ B regulates MDR1 expression

Since the expression of MDR1 is known to be regulated by NF- κ B, we investigated whether the effect of HQ on the expression of MDR1 is mediated by the NF- κ B pathway. We therefore examined the effect of ectopic expression of the NF- κ B subunit p65 on the levels of MDR1 in BMSCs. Our results showed that p65 dose-dependently upregulated the expression of MDR1 in BMSCs (Fig. 6A). To confirm that the expression of MDR1 is regulated directly by the NF- κ B pathway, we performed a luciferase assay by transfecting cells with a luciferase reporter gene driven by the MDR1 promoter region together with increasing amounts of the p65 protein. The results showed that the p65 subunit dose-dependently increased luciferase activity, indicating the activation of the MDR1 promoter by NF- κ B (Fig. 6B).

Discussion

In the present study, we showed that HQ decreased viability and induced apoptosis in BMSCs in a dose and time dependent manner. HQ downregulated MDR1 mRNA and protein

expression, and MDR1 overexpression attenuated the effect of HQ on cell viability and the induction of apoptosis, whereas MDR1 knockdown enhanced the effect of HQ at specific concentrations. We found that HQ regulates MDR1 expression by modulating the activity of the NF- κ B pathway. Taken together, our results suggest that benzene cytotoxicity is mediated by the downregulation of MDR1 via the modulation of the NF- κ B pathway.

Benzene toxicity has been demonstrated even in response to low levels of occupational exposure, affecting white blood cell and platelet counts as well as significantly affecting the colony forming ability of myeloid stem and progenitor cells [27]. Despite the known association between benzene exposure and the development of leukemia, the exact mechanism remains elusive and other environmental or genetic factors contributing to leukemia etiology need to be identified. The inhibition of MDR1 has been suggested as a potential mechanism causing the accumulation of benzene intermediate compounds in the bone marrow, and thus an underlying cause of the hematotoxicity of benzene [28]. MDR1 was identified three decades ago as a drug export pump whose expression was induced in response to chemical drug application, leading to multidrug resistance. Since then, MDR1 has been studied extensively because of the role of its encoded protein P-gp in extruding drugs from cancer cells, thereby conferring resistance to chemotherapeutic agents. P-gp expression is a marker of chemoresistance in leukemias, lymphomas, and osteosarcomas among several malignancies [29-31]. We showed that MDR1 overexpression attenuated the effect of HQ on decreasing BMSC viability and inducing apoptosis, and HQ downregulated MDR1 expression in BMSCs, indicating that the cytotoxicity of HQ may be mediated by the downregulation of MDR1.

Numerous signals are involved in the regulation of MDR1, among them the transcription factor NF- κ B [28]. A binding site for NF- κ B was identified in the MDR1 promoter and shown to play a role in its transcriptional activation [32, 33]. NF- κ B plays an antiapoptotic role in many cell types, and inhibition of NF- κ B activity has been shown to induce cell death [11]. However, NF- κ B can also play a proapoptotic role in response to chemotherapeutic drugs, and induction of p53 induces apoptosis through the activation of NF- κ B [34, 35]. Inhibition of NF- κ B was shown to increase the cellular response to cytotoxic agents in different tumors [36-38]. Activation of NF- κ B upregulates the expression of MDR1 in human hepatoma cells [39], and binding of NF- κ B to the MDR1 promoter induces drug resistance by upregulating the expression of P-gp in colon cancer cells [11]. Clitocine, a natural compound, was recently shown to suppress P-gp associated multidrug resistance by downregulating NF- κ B in human hepatoma cells [40]. These studies support the role of NF- κ B in the modulation of MDR1 expression and drug resistance and thus the results of the present study. Here, we showed that HQ inhibited NF- κ B activity and ectopic expression of the NF- κ B p65 subunit upregulated MDR1, suggesting that the cytotoxic effect of HQ is mediated by the downregulation of MDR1 via the NF- κ B pathway.

In the present study, we examined potential signaling pathways mediating the effect of benzene and its metabolites on the viability of BMSCs. However, the cytotoxic effect of HQ could be mediated by different pathways, and the relationship between TNF- α , MDR1 and NF- κ B could involve crosstalk between these and other as yet unidentified pathways triggered by stimulation by a toxic compound. Further study is necessary to elucidate the mechanisms underlying the cytotoxic effect of benzene and the interplay between the molecules involved. In addition, other mechanisms potentially mediating the effect of HQ on BMSCs should be investigated, such as the involvement of caspases, as the induction of cell death by benzene and its metabolites has been shown to be mediated by the modulation of several genes including Bcl-2, Bax, caspases 9, 11 and 12, ELK-member of ETS oncogene family and Fau [41, 42], as well as by oxidative DNA damage resulting from the production of reactive oxygen species in response to HQ [43].

In conclusion, we showed that the benzene metabolite HQ decreases cell viability and induces apoptosis in BMSCs by downregulating the expression of MDR1 via a mechanism involving the NF- κ B pathway.

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Disclosure Statement

The authors have no conflict of interest.

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