Tetrahydroxyquinone induces apoptosis of leukemia cells through diminished survival signaling

Alexandre D. Martins Cavagis, Carmen Veríssima Ferreira, Henri H. Versteeg, Cristiano Fernandes Assis, Carina L. Bos, Sylvia A. Bleuming, Sander H. Diks, Hiroshi Aoyama, and Maikel P. Peppelenbosch

Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brasil; Department of Experimental Internal Medicine, Academic Medical Center (AMC), University of Amsterdam, The Netherlands; Department of Cell Biology, University of Groningen, Groningen, The Netherlands

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Objective. Tetrahydroxyquinone is a molecule best known as a primitive anticitcataract drug but is also a highly redox active molecule that can take part in a redox cycle with semiquinone radicals, leading to the formation of reactive oxygen species (ROS). Its potential as an anticancer drug has not been investigated.

Methods. The effects of tetrahydroxyquinone on HL60 leukemia cells are investigated using fluorescein-activated cell sorting–dependent detection of phosphatidylserine exposure combined with 7-amino-actinomycin D exclusion, via Western blotting using phosphospecific antibodies, and by transfection of constitutively active protein kinase B.

Results. We observe that in HL60 leukemia cells tetrahydroxyquinone causes ROS production followed by apoptosis through the mitochondrial pathway, whereas cellular physiology of normal human blood leukocytes was not affected by tetrahydroxyquinone. The antileukemic effect of tetrahydroxyquinone is accompanied by reduced activity of various antiapoptotic survival molecules including the protein kinase B pathway. Importantly, transfection of protein kinase B into HL60 cells and thus artificially increasing protein kinase B activity inhibits tetrahydroxyquinone-dependent cytotoxicity.

Conclusion. Tetrahydroxyquinone provokes cytotoxic effects on leukemia cells by reduced protein kinase B–dependent survival signaling followed by apoptosis through the mitochondrial pathway. Thus, tetrahydroxyquinone may be representative of a novel class of chemotherapeutic drugs, inducing apoptosis in cancer cells through diminished survival signaling possibly as a consequence of ROS generation. © 2006 International Society for Experimental Hematology. Published by Elsevier Inc.

Chemotherapy for the treatment of some types of neoplastic disease has been one of the success stories of medicine. However, the chemotherapeutic treatment outcome of most adult acute myeloid leukemia (AML) remains unacceptable [1]. Among AMLs, acute promyelocytic leukemia can be successfully treated with all-trans-retinoic acid (ATRA). The development, however, of resistance to a wide spectrum of cytotoxic drugs frequently impedes the successful treatment of AML either in the beginning of disease or following primary or subsequent relapses. Moreover, ATRA resistance in acute promyelocytic leukemia is rare but markedly increases in frequency after relapses from chemotherapy-induced clinical remission [2,3]. Hence, novel avenues for the treatment of AML are required.

It is now generally recognized that the reactive oxygen species (ROS) play an important role as regulatory mediators in signaling processes [4]. Accordingly, it has now been shown that a multitude of physiological processes are under the direct control of ROS, the most important being the regulation of vascular tone, the sensing of oxygen...
tension, the enhancement of leukocyte signal transduction, and the induction of apoptosis, the latter as an essential component of the tumor necrosis factor \( \alpha \)-dependent signal transduction [5–9]. Therefore, ROS generation is an important element in the control of cellular biochemical processes. In addition, ROS generation is important for inducing cytotoxicity in cancer cells, but the molecular mechanism underlying these cytotoxic effects remains unclear, hampering the development of more effective drugs.

ROS formation depends on the univalent reduction of triplet-state molecular oxygen [10]. This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or nonenzymatic by redox-reactive compounds such as tetrahydroxyquinone [11–13]. Superoxide dismutases convert superoxide enzymatically into hydrogen peroxide or nonenzymatically into nonradical species, hydrogen peroxide and singlet oxygen. Hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. This latter enzyme oxidizes glutathione to glutathione disulfide, which can be converted back to glutathione by glutathione reductase in an NADPH-consuming process [14,15]. Thus, the biochemistry of ROS generation is relatively well understood.

The biological effects of ROS production, however, are less clear. At high concentrations ROS are dangerous for living organisms, damaging virtually all cellular constituents [10]. Nevertheless, at moderate concentrations, ROS play important roles in the control of various cellular functions and cellular generation of ROS is actively induced under various conditions [4,10]. The archetypal example is NADPH oxidase activation upon immune stimulation of the phagocytic cells of the myeloid lineage, resulting in ROS production, which, apart from its bactericidal function, is also instrumental for the induction of proinflammatory gene transcription. Hence, phagocyte ROS production is pivotal for proper function of the innate immune system [16–18].

Various studies have shown that ROS-producing drugs can exert important cytotoxic effects in leukemia cells, although the molecular details by which such drugs mediate cancer cell death remain obscure [15]. This consideration prompted us to test the effect of as a cytotoxic agent for HL60 leukemia cells. Tetrahydroxyquinone is a compound best known as a primitive antitumor drug but is expected to act as a redox active benzoquinone [13]. In the present study we observe that tetrahydroxyquinone indeed efficiently induces ROS generation, which is in turn responsible for HL60 cell apoptosis through the mitochondrial pathway. This apoptosis is accompanied by reduced activity of the antiapoptotic protein kinase B (PKB) [14] and nuclear factor (NF)-\( \kappa \)B pathways [15] while concomitantly specific activation of Jun-N-terminal kinase and protein phosphatases (PPs) is observed [16]. Importantly, forced expression of PKB counteracts the effect of tetrahydroxyquinone (THQ) on HL60 cell apoptosis. Thus the diminished survival signaling is essential for tetrahydroxyquinone-dependent cytotoxicity, and tetrahydroxyquinone may be representative of a novel class of chemotherapeutic drugs, inducing apoptosis in cancer cells through diminished survival signaling. The tetrahydroxyquinone pathways mediating this effect involve at least in part ROS generation.

Materials and methods

Cell line and reagents

HL60 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Polyclonal antibodies anti-caspase 3, anti-breakpoint cluster-2 (BCL2)/BCL-XL-associated death promoter (BAD), anti-phospho-BAD, anti-phospho-p38 mitotube-associated protein kinase (MAPK), anti-phospho-inhibitory factor (I)-\( \kappa \)B, anti-IxB, anti-phospho-protein kinase C (PKC) del- ta, anti-phospho-PKB, anti-phospho-raf-1 murine leukemia viral oncogene (Raf), anti-phospho-p42/44 MAPK, anti-phospho-JNK, anti-phospho-cAMP responsive element binding protein, anti-rabbit and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies against phospho-PP 2A, phosphosyrosine, phosphothreonine, and NF-\( \kappa \)B (p50 and p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tetrahydroxyquinone was purchased from Sigma Chemical Company (Zwijndrecht, The Netherlands).

Leukocyte culture

Human blood was collected from healthy donors and human peripheral blood mononuclear cells were isolated by Ficoll/Hypaque gradient centrifugation. Leukocytes were cultured at the same conditions described for HL60 cells, the only difference being the addition of 5 \( \mu \)g/mL phytohemaglutinin in each well. Cells were plated at density of 1 \( \times \) 10^6 plating/mL in a 24-well plate. The medium was removed 48 hours after cell seeding and replaced with medium containing tetrahydroxyquinone.

Cell culture and viability assays

HL60 cells were routinely grown in suspension in Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 \( \mu \)g/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS), at 37°C in a 5% CO_2 humidified atmosphere. In all experiments 3 \( \times \) 10^5 cells/mL were seeded and, after 72 hours, treated with different tetrahydroxyquinone concentrations for the specified periods of time.

Cell viability was assessed based on trypan blue dye exclusion and three additional parameters: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, protein phosphatase activity, and determination of the total protein amount.

**MTT reduction assay**

The medium containing tetrahydroxyquinone was removed and 1.0 mL of MTT solution (0.5 mg MTT/mL of culture medium) was added to each well. After incubation for 4 hours at 37°C, the medium was removed and the formazan released by solubilization in 1.0 mL of ethanol. The plate was shaken for 5 minutes on a plate shaker and the absorbance measured at 570 nm [19,20].
Protein phosphatase assay
The phosphatase extract was obtained by lysis of the cells with acetate buffer 0.1 mM (pH 5.5). Then, the enzyme activity was measured in a reaction medium (final volume, 1.0 mL) containing 100 mM acetate buffer (pH 5.5), 5.0 mM p-nitrophenyl phosphate (pNPP), and cell extract enzyme. After a 30-minute incubation at 37°C, the reaction was stopped by adding 1.0 mL of 1.0 M NaOH. The amount of pNPP released was measured at 405 nm [21].

Protein quantification
Protein concentration was determined using a Lowry protein assay. An equal volume of 2× loading buffer [100 mM Tris–HCl (pH 6.8), 200 mM dithiotheritol (DTT), 4% SDS, 0.1% bromophenol blue, and 20% glycerol] was added to samples, which were subsequently boiled for 10 minutes. Afterward, cell extracts were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to polyvinylidene-fluoride membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-hydroxymethylaminomethane-buffered saline (TBS)-polyoxyethyleneorbitan mono-oleate (Twee) 20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibodies, at 1:2000 dilutions, in blocking buffer for 1 hour. Detection was performed using enhanced chemiluminescence.

Mitochondrial extract preparation (cytochrome c release)
For cytochrome c analysis, cells were washed with ice cold PBS and resuspended in lysis buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes)–NaOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, and protease inhibitors [1 μg/mL apro tin, 10 μg/mL leupeptin, and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluorid-hydrochloride]. After incubation on ice for 1 hour, the lysate was centrifuged at 14,000g for 15 minutes. Then the supernatant was removed in an SDS-PAGE as described.

Annexin V and 7-amino-actinomycin D assays
Control and tetrahydroxyquinone-treated cells were collected and resuspended in 1× binding buffer (0.01 M Hepes/NaOH, pH 7.4, 0.14 mM NaCl, and 2.5 mM CaCl₂) at a concentration of 1×10⁶ cells/mL. Subsequently, 100 μL of cell suspension was transferred to a 5-mL tube and 10 μL of Annexin V fluorescein isothiocyanate (5 μL) and 7-amino-actinomycin D (7-AAD) was added. The cells were incubated at room temperature for 15 minutes, after which 400 μL of 1× binding buffer was added and apoptosis analyzed by flow cytometry.

Transient transfection of HL60 cells
HL60 cells (4×10⁶) were transfected with 0.4 μg of a plasmid expressing group-specific antigen polypeptide (GAG)-PKB (a kind gift of Dr. Paul Coffer), a constitutive form of PKB. Cells were cotransfected with 0.1 μg of a pUT-galactosidase to normalize for transfection efficiency. After transfection, the cells were cultured for 24 hours, harvested, lysed in commercially available reporter lysis buffer (Promega, Madison, WI), and β-galactosidase activity was determined using chlorophenol red-β-D-galactopyranoside (Roche, Ingelheim, Germany) as substrate.

Transfected cells were treated with tetrahydroxyquinone for 24 hours and the cell viability assessed by MTT reduction. The expression and activation of PKB was verified by western blotting.

Statistical evaluation
All experiments were performed in triplicate and the results shown in the figures represent the mean and standard deviation. Cell viability data were expressed as the means ± standard errors of three independent experiments carried out in triplicates. Data from each assay were analyzed statistically by analysis of variance followed by a Dunnett’s test. Multiple comparisons among group mean differences were checked with Tukey post hoc test. Differences were considered significant when the p value was less than 0.05. Western blottings represent three independent experiments.
Results

Tetrahydroxyquinone is cytotoxic for HL60 leukemia cells

As evidence has been presented that the redox state-altering agents are potent cytotoxic agents in leukemia cells, although acting through as yet unknown molecular mechanisms [24,25], we decided to test the possible cytotoxic effects of tetrahydroxyquinone on HL60 cells. To this end, HL60 were treated with various concentrations of tetrahydroxyquinone for 24 hours and cell viability was determined using total protein content, cellular phosphatase activity, or mitochondrial function (MTT reduction, Fig. 1A) as a measure. It appeared that using either measure, tetrahydroxyquinone was highly cytotoxic to the leukemia cells, the apparent IC₅₀ of tetrahydroxyquinone-induced cytotoxicity being similar whether assessed by total protein content (IC₅₀ 20 µM), phosphatase activity (IC₅₀ 40 µM), or by MTT assay (IC₅₀ 45 µM). Importantly, when healthy human leukocytes were exposed to tetrahydroxyquinone no apparent toxicity was observed, even at concentrations 10 times as high as the IC₅₀ for leukemia cells, when assayed by protein content, phosphatase activity, or MTT (Fig. 1C). Thus tetrahydroxyquinone is specifically cytotoxic for HL60 leukemia cells but not for the corresponding untransformed counterparts.

Tetrahydroxyquinone-dependent ROS generation mediates cytotoxicity

Tetrahydroxyquinone is a highly redox active molecule, expected to induce the formation of ROS by taking part in a redox cycle with semiquinone radicals. We decided to investigate whether the tetrahydroxyquinone-mediated cytotoxic effects are mediated by ROS generation. In agreement with a role of ROS generation in tetrahydroxyquinone cytotoxicity, we observed that the compound substantially increases the cellular levels of ROS, as determined by

Figure 1. Effect of tetrahydroxyquinone on HL60 cell viability versus effects on untransformed cells. HL60 cell viability was evaluated using three different parameters (protein content, MTT reduction, and phosphatase activity) after treatment with tetrahydroxyquinone for 24 hours, in the absence (A) or presence of the ROS scavenger glutathione (10 mM; B). The lack of effect of tetrahydroxyquinone on normal peripheral blood mononuclear cell leukocytes is also depicted (C). Each point represents the mean ± standard deviation of three independent experiments.
dichlorofluorescein diacetate-dependent spectrophotometry (Fig. 2). Importantly, treatment with reduced glutathione or N-acetyl-L-cysteine abolished the capacity of HL60 cells to react to tetrahydroxyquinone with ROS production (Fig. 2). This allowed us to test the importance of tetrahydroxyquinone-induced ROS formation for its cytotoxic effects, and a significant rightward shift of the dose-response curve with respect to tetrahydroxyquinone-induced cytotoxicity was observed in the presence of ROS generation inhibitors whether assessed by total protein content (IC50 from 20 μM to 45 μM), phosphatase activity (IC50 from 40 μM to 140 μM), or MTT assay (IC50 from 45 μM to 140 μM; Fig. 1A and B). Thus tetrahydroxyquinone is an efficient inducer of ROS production in HL60 leukemia cells and ROS generation is essential for the cytotoxic effect of this compound.

Tetrahydroxyquinone induces cell death by apoptosis

Generally speaking cell death is brought about either via necrosis or via apoptosis. The former process is associated with relatively large damage to the surrounding tissue. The latter is associated with controlled elimination of cancer cells. Thus for the possible treatment of leukemia cytotoxic compounds should preferentially act via apoptosis. To address the question whether tetrahydroxyquinone induces cell death via apoptosis we measured three cellular processes associated with apoptosis rather than necrosis: caspase 3 activation, DNA fragmentation, and phosphatidylserine exposure. It appeared that tetrahydroxyquinone efficiently
activates caspase 3 (Fig. 3A) in concentration in excess of 25 μM, stimulates DNA fragmentation at the same concentration (Fig. 3B), and provokes phosphatidylserine exposure (Fig. 3C) when applied in a concentration of 25 μM or more. Importantly, induction of apoptosis as measured by DNA fragmentation was not detected when the formation of ROS was blocked (Fig. 3B). Thus apoptosis is the main route to cell death in tetrahydroxyquinone-treated cells.

**JNK and protein phosphatases activation in HL60 cells**

To investigate the molecular mechanism underlying tetrahydroxyquinone-dependent apoptosis induction, we studied the activation status of the MAP kinase family, since this family of kinases is well known to be involved in the control of a variety of cell survival-controlling pathways [23]. In myeloid leukemia cell lines the p42/p44 MAP kinase cascade positively regulates differentiation into the monocytic lineage and is instrumental for phorbol ester-dependent differentiation of this cell type, while inhibition of the JNK has been implicated in 1,25-dihydroxyvitamin D3-dependent HL60 cell differentiation. Conversely, in HL60 cells JNK activation is linked to apoptosis [24,25]. We observed that tetrahydroxyquinone treatment strongly activated JNK. Unexpectedly, only a modest increase in the phosphorylation of p38 MAP kinase with little effect on the activation state of p42/44 MAP kinase was observed, even at concentrations as high as 100 μM (Fig. 4A). Thus, tetrahydroxyquinone-induced changes in the activation of MAP kinase family members are discordant with an effect on differentiation induction and is consistent with an effect on apoptosis in this cell type [24,25].

Despite the induction of oxidative stress by tetrahydroxyquinone, unusual activations of both protein tyrosine phosphatases and protein serine/threonine phosphatases coinciding with the activation of PP2A were observed (Fig. 4B).

**Tetrahydroxyquinone-induced apoptosis coincides with activation of the mitochondrial pathway through diminished PKB activation**

Apoptosis can be executed through two basic signaling pathways: the extrinsic pathway and the mitochondrial intrinsic pathway. We observed that application of tetrahydroxyquinone induced the release of cytochrome c from the mitochondria at concentration as low as 25 μM (Fig. 5), demonstrating that tetrahydroxyquinone activates the mitochondrial pathway. Importantly, the phosphorylation of Ser112 Bad (which leads to cell survival by inhibiting the mitochondrial pathway) was concomitantly decreased. In agreement, tetrahydroxyquinone treatment also caused increase of phosphorylation of Ser473 in PKB (the Bad kinase for Ser112), demonstrating that tetrahydroxyquinone signaling reduces activity of this antiapoptotic kinase and, consequently, leads to apoptosis. The importance of mitochondria for apoptosis induction by tetrahydroxyquinone was also confirmed by a dramatic increase in total Bad protein levels (Bad phosphorylation is followed by its ubiquitination and proteolysis and mediates the inhibitory effect of PKB on apoptosis through the mitochondrial pathway). In addition, inhibition of two other kinases involved in the survival signaling, Raf and PKC delta, reinforced our hypothesis that tetrahydroxyquinone inhibits cell survival signaling pathways (Fig. 5).

Furthermore, additional evidence for survival pathway inhibition by tetrahydroxyquinone was provided through down-regulation of NF-κBp65 and a decrease of phosphorylated IkB (Fig. 6). Independent support for this notion was obtained in experiments in which the PKB activity was artificially increased by transfection with GAG-PKB, a constitutive active form of PKB expression construct. As shown in the Figure 7, HL60 cells transfected with PKB became resistant to tetrahydroxyquinone, even when treated with 500 μM of the compound.

**Figure 4.** Effect of tetrahydroxyquinone on MAPK and PP activities. The phosphorylation and total protein level of MAPK family members (A), phosphoprotein profiles (employing anti-phospho-amino acid antibodies and phospho-PP2A (B) were evaluated by immunoblotting. The panel shows tetrahydroxyquinone-induced chances in phosphorylation state (see arrows).
Discussion

It is now generally recognized that novel compounds are called for treating leukemic disease. This consideration prompted us to investigate the consequences of tetrahydroxyquinone on HL60 leukemia cells, a compound most readily known as a primitive anticataract drug, but also expected to act as a redox active molecule. In agreement with the latter notion, we observed that tetrahydroxyquinone led to ROS production that coincided with apoptosis through the mitochondrial pathway and corresponded with reduced activity of various antiapoptotic survival molecules including the PKB pathway in HL60 leukemia cells but not their untransformed counterparts. Importantly, transfection of PKB into HL60 cells and thus artificially increasing PKB activity inhibited ROS-dependent cytotoxicity. We concluded that the remarkable cytotoxic effects of tetrahydroxyquinone on HL60 leukemia cells are dependent on reduced PKB-dependent survival signaling followed by apoptosis through the mitochondrial pathway, probably as a consequence of tetrahydroxyquinone-dependent ROS generation. Thus tetrahydroxyquinone may be representative of a novel class of chemotherapeutic drugs, inducing apoptosis in cancer cells through diminished survival signaling as a consequence of ROS production. Proof of this notion awaits in vivo experiments in which the direct potential of tetrahydroxyquinone as an anticancer drug is directly demonstrated.

Increased ROS production by NADPH oxidase upon macrophage activation is well established. Earlier studies have shown that such ROS production upon immune stimulation of the phagocytic cells, apart from its bactericidal function, is also instrumental for the induction of proinflammatory gene transcription [16–18]. Among the cellular responses to macrophage activation is also cell death, a response probably required to rid the body from cells damaged by the immune response. HL60 cells share important characteristics with monocytes and macrophages, and the data obtained in the present study may indicate that this apoptosis is a direct consequence of ROS production, followed by reduced survival signaling and activation of mitochondrial pathway of cell death, which is also important in the induction of apoptosis following macrophage activation. Final proof of this notion, however, awaits experiments in which activation-induced cell death is investigated in the presence and absence of glutathione and N-acetyl-L-cysteine.

The molecular mechanisms by which ROS participate in inflammatory gene expression are obscure at best. In the

![Figure 5](image1.png)

**Figure 5.** Participation of mitochondrial pathway of apoptosis in tetrahydroxyquinone-dependent cytotoxicity. Cells were treated with tetrahydroxyquinone as indicated and lysates were prepared as appropriate (see Methods) and analyzed by immunoblotting for PKB, phospho-Bad, and total Bad protein levels.

![Figure 6](image2.png)

**Figure 6.** Diminished levels of NF-κB by tetrahydroxyquinone. HL60 cells were treated with tetrahydroxyquinone as indicated and the level of both subunits of NF-κB (p65 and p50) and its phosphorylated inhibitory protein (IκB) were analyzed by Western blotting.

![Figure 7](image3.png)

**Figure 7.** Viability analysis of HL60 cells transfected with PKB. HL60 cell viability was evaluated using MTT assay after treatment with tetrahydroxyquinone for 24 hours in cell transfected with a PKB expression construct (filled circles) or empty vector (open circles).
References


