Mechanisms of drug-induced apoptosis in human leukemia
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Tetrahydroxyquinone induces apoptosis of leukemia cells through diminished survival signaling

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ABSTRACT

Objective
Tetrahydroxyquinone is a molecule best known as a primitive anti-cataract drug, but is also a highly redox active molecule which 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 leukaemia cells are investigated using FACS-dependent detection of phosphatidylserine exposure combined with 7-amino-actinomycin D (7-AAD) exclusion, via Western blotting using phosphospecific antibodies, and by transfection of constitutively active protein kinase B (PKB).

Results
We observe that in HL60 leukaemia 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 anti-leukaemic effect of tetrahydroxyquinone is accompanied by reduced activity of various anti-apoptotic 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 leukaemia cells by reduced protein kinase B-dependent survival signalling 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 signalling possibly as a consequence of ROS-generation.

Keywords: HL60 cells; tetrahydroxyquinone; protein phosphatases; MAPKs; oxidative stress.
INTRODUCTION

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 recognised that the reactive oxygen species (ROS) play an important role as regulatory mediators in signalling 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 tumour necrosis factor α-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 non-enzymatically into non-radical 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 ROS production which, apart from its bactericidal function, is also instrumental for the induction of pro-inflammatory 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 leukaemia 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 leukaemia cells. Tetrahydroxyquinone is a compound best known as a primitive anti-cataract drug, but expected to act as a redox active benzoquinone [13]. In the present study we observe that tetrahydroxyquinone indeed efficiently induces ROS generation in turn responsible for HL60 cell apoptosis through the mitochondrial pathway. This apoptosis is accompanied by reduced activity of the anti-apoptotic PKB [14] and nuclear factor (NF)-κB 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 on HL60 cell apoptosis. Thus the diminished survival signalling 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 signalling. 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 (ATCC, Rock-ville, MD). Polyclonal antibodies anti-caspase 3, anti-BAD, anti-phospho-BAD, anti-phospho-p38 MAPK, anti-phospho-IκB, anti-IκB, anti-phospho-PKC delta, anti-phospho-PKB, anti-phospho-Raf, anti-phospho-p42/44, anti-phospho-JNK, anti-phospho-CREB, anti-rabbit and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Techno-logy (Beverly, MA). The antibodies against phos-pho-PP2A, phosphotyrosine, phospho-threonine and NF-κB (p50 and p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tetrahydroxy-quinone was purchased from Sigma Chemical Company.

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 was the addition of 5 µg/ml phytohemaglutinin in each well. Cells were plated at density of 1 x 10^6 plating/ml in 24-well plate. The medium was removed 48h after cell seeding and replaced with medium containing tetrahydroxyquinone.

Cell Culture and viability assays

HL60 cells were routinely grown in suspension in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum, at 37°C in a 5% CO₂ humidified atmosphere. In all experiments, 3 x 10^5 cells/ml were seeded and, after 72 h, treated with different tetra-hydroxyquinone concentrations for the specified periods of time.

Cell viability was assessed based on trypan blue dye exclusion and three additional parameters: MTT reduction, protein phosphatase activity and determination of the total protein amount.
**MTT reduction assay**

The medium containing tetrahydroxyqui-none 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 h at 37°C, the medium was removed and the formazan released by solubilisation in 1.0 ml of ethanol. The plate was shaken for 5 min on a plate shaker and the absorbance was measured at 570 nm (15, 16).

**Protein phosphatase assay**

The phosphatase extract was obtained by lysing 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 pNPP and cell extract enzyme. After a 30 min incubation at 37°C, the reaction was stopped by adding 1.0 ml of 1.0 M NaOH. The amount of pNP released was measured at 405 nm (17).

**Protein quantification**

Protein concentrations were determined by a modification of the Lowry method as described by Hartree (18).

**Reduced glutathione determination**

The concentration of reduced glutathione in HL60 cells was determined after treatment of the cells for 24 h. The cells (3×10^5/ml) were washed with physiological solution and lysed with water; 3 ml of precipitant solution (1.67 g glacial metaphosphoric acid, 0.2 g ethylenediaminetetraacetic acid (EDTA) and 30 g NaCl in 100 ml MilliQ water) was added to the lysate (2 ml). After 5min, this mixture was centrifuged and 0.4 ml of the supernatant was added to 1.6 ml of reaction medium (0.2 M Na_2HPO_4 buffer, pH 8.0; 0.5 mM DTNB dissolved in 1% sodium citrate). Subsequently, the absorbance of the product (NTB) was measured at 412 nm and reduced glutathione concentration calculated using the extinction coefficient \( E = 13.6 \text{ mol}^{-1} \text{ cm}^{-1} \) (19).
Spectrofluorometric determination of ROS

HL60 cells were treated with 100 µM tetra-hydroxyquinone or the combinations tetrahydroxy-quinone/10 mM glutathione and tetrahydroxyqui-none/15 mM N-acetyl-L-cysteine for 24 h in RPMI 1640 (without phenol red) containing 5% serum. Afterwards, the cells (2x10⁶/10 ml) were washed with PBS and resuspended in RPMI 1640 (without phenol red, with 5% FBS) containing 20 µM DCFH-DA (dichlorofluorescein diacetate). After 30 min of incubation at 37°C, the cells were washed three times and resuspended in RPMI 1640 (without phenol red, with 5% FBS). The fluorescence of the suspension was measured using a RF-5300 PC Shimadzu spectrofluorometer with excitation at 485 nm and emission at 530 nm.

DNA fragmentation analysis

The cell pellets (5x10⁶) were lysed in 0.5 ml of lysis buffer containing 5 mM Tris-HCl, 20 mM ethilenediaminetetraacetic acid (EDTA) and 0.5% Triton X 100. After centrifugation at 1,500 x g for 10 min, the pellets were resuspended in 250 µL of lysis buffer and, to the supernatants (S), 20 µL of 6 M perchloric acid was added. Then, 500 µL of 10% trichloroacetic acid (TCA) were added to the pellets (P), the samples were centrifuged for 10 min at 5,000 rpm and the pellets were resuspended in 250 µL of 5% TCA followed by incubation at 100°C for 15 min. Subsequently, to each sample, 500 µL of solution (15 mg/ml DPA in glacial acetic acid), 15 µL/ml of sulfuric acid and 15 µg/ml acetaldehyde were added and incubated at 37 °C for 18 h (20). The proportion of fragmented DNA was calculated from the absorbance at 594 nm using the following formula:

\[
\text{Fragmented DNA (\%) = 100 x (amount of the fragmented DNA in the supernatant) / (amount of the fragmented DNA in the supernatant + amount DNA in the pellets).}
\]

Western blotting

Cells (3 x 10⁷) were lysed in 200 µL of lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na₃VO₄, 1 mM NaF and protease inhibitors (1 µg/ml aprotinin and 1 µg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride)] for 2 h on ice. Protein extracts were cleared by


centrifugation and protein concentrations were determined using a Lowry protein assay. An equal volume of 2X sodium dodecyl sulfate (SDS) gel loading buffer [100 mM Tris–HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol] was added to the samples, which were subsequently boiled for 10 min. Afterwards, cell extracts were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)-Tween 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 1h. Detection was performed using enhanced chemiluminescence (ECL).

**Mitochondrial extract preparation (cytochrome c release)**

For cytochrome c analysis, the cells were washed with ice cold PBS and resuspended in lysis buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM Hepes–NaOH, pH 7.4, 50 mM KCl, 5mM EGTA, 2mM MgCl₂, 1 mM DTT, and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin and 1mM 4-(2-amino-ethyl)-benzolsulfonyl-fluoride-hydrochloride). After incubation on ice for 1 h, the lysate was centrifuged at 14,000 x g for 15 min. Then, the supernatant was resolved by SDS-PAGE.

**Annexin V and 7-amino-actinomycin D assays**

Control and tetrahydroxyquinone-treated cells were collected and resuspended in 1X binding buffer (0.01 M Hepes/NaOH, pH 7.4, 0.14 mM NaCl and 2.5 mM CaCl₂) at a concentration of 1 x 10⁶ cells/ml. Subsequently, 100 µl of cell suspension were transferred to a 5 ml tube and Annexin V FITC (5 µl) and 7-amino-actinomycin D (7-AAD) - (10 µl) were added. The cells were incubated at room temperature for 15 min, after which 400 µl of 1X binding buffer was added and apoptosis then analyzed by flow cytometry.
Transient transfection of HL60 cells

HL60 cells (4 x 10^6) were transfected with 0.4 µg of a plasmid expressing GAG-PKB, 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 24h, harvested, lysed in commercially available reporter lysis buffer (Promega) and β-galactosidase activity was determined using chloro-phenol red-β-D-galactopyranoside (Roche) as substrate.

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

Statistical evaluation

All experiments were performed in triplicate and the results shown in the graphs 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 ANOVA 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 leukaemia cells

As evidence has been presented that the redox state-altering agents are potent cytotoxic agents in leukaemia cells, although acting through as yet unknown molecular mechanisms [23, 24], 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 leukaemia cells, the apparent IC50 of
tetrahydroxyquinone-induced cytotoxicity being similar whether assessed by total protein content (IC$_{50}$ 20 µM), phosphatase activity (IC$_{50}$ 40 µM), or by MTT assay (IC$_{50}$ 45 µM).

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 h, in the absence (A) or presence of the ROS scavenger glutathion (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.
Importantly, when healthy human leukocytes were exposed to tetrahydroxy-quinone no apparent toxicity was apparent, even at concentrations 10 times as high as the IC$_{50}$ for leukaemia cells, when assayed by protein content, phosphatase activity, or MTT (fig 1C). Thus tetrahydroxyquinone is specifically cytotoxic for HL60 leukaemia 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 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 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 (IC$_{50}$ from 20 µM to 45 µM), phosphatase activity (IC$_{50}$ from 40 µM to 140 µM), or by MTT assay (IC$_{50}$ from 45 µM to 140 µM) (Fig. 1A,B). Thus tetrahydroxyquinone is an efficient inducer of ROS production in HL60 leukaemia cells and ROS generation is essential for the cytotoxic effect of this compound.
Figure 2. Tetrahydroxyquinone causes production of reactive oxygen species. The production of reactive oxygen metabolites was determined using dichlorofluorescein diacetate-loaded cells and a spectrofluorometer (excitation at 485 nm and emission at 530 nm). 1 - Non-treated HL60 cells (control); 2 – tetrahydroxyquinone (100 µM) - treated cells; 3 - HL60 cells pre-treated with N-acetyl-L-cysteine (10 mM) and 4 - HL60 cells pre-treated with reduced glutathione (10 mM) for 30 min. For conditions 3 and 4, the cultures were further incubated with 100 µM tetrahydroxyquinone for 24h. Afterwards, a 30 min incubation with DCFH-D was carried out and the fluorescence intensity was recorded by spectrofluorometry. Results were expressed as the relative fluorescence intensity with respect to untreated cells. 5 - As a positive control - H$_2$O$_2$-treated cells were used to monitor the level of ROS. Bars represent mean ± standard deviation.

**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 leukaemia 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.
**Figure 3. Apoptosis induction by tetrahydroxyquinone.** (A) Cells were treated with tetrahydroxyquinone as indicated and lysates were resolved by immunoblotting as described in methods to assess the level of active caspase 3. (B) The samples were prepared as described by Zhu and collaborators (23) and the proportion of fragmented DNA was calculated from absorbance at 594 nm using the approach described in the method section. The bars represent the mean ± standard deviation of three experiments. (C) Cell samples were prepared as described in Methods and Annexin V-positive, 7-AAD-positive and Annexin V/7-AAD-positive populations were analyzed by flow cytometry.

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 provoke 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.
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 [25]. In myeloid leukemia cell lines the p42/p44 MAP kinase cascade positively regulates differentiation into the monocyte lineage and is instrumental for phorbolester-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 [23, 24]. 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 [23, 24].

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).
Figure 4. Effect of tetrahydroxyquinone on MAP kinase and protein phosphatase 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 tetrahydroxyquine-induced changes in phosphorylation state (see arrows).
Tetrahydroxyquinone-induced apoptosis coincides with activation of the mitochondrial pathway through diminished protein kinase B activation

Apoptosis can be executed through two basic signalling 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 Bad Ser112 (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 protein kinase B (the Bad kinase for Ser112), demonstrating that tetrahydroxyquinone signalling reduces activity of this anti-apoptotic kinase and, consequently, leads to apoptosis.

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 analysed by immunoblotting for PKB, phospho-Bad and total Bad protein levels.
THQ induces apoptosis of leukemia cells through diminished survival signaling

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 protein kinase B on apoptosis through the mitochondrial pathway). In addition, inhibition of two other kinases involved in the survival signalling: Raf and PKC delta, reinforced our hypothesis that tetrahydroxyquinone inhibits cell survival signalling 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 IκB (Fig. 6).

![Figure 6](image)

**Figure 6. Diminished levels of nuclear factor kappa B 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 analysed by western blotting.

Independent support for this notion was obtained in experiments in which the protein kinase B activity was artificially increased by transfection with GAG-protein kinase B, a constitutive active form of protein kinase B, expression construct. As it has been shown in the Figure 7, HL60 cells transfected with protein kinase B became resistant to tetrahydroxyquinone, even when treated with 500 μM of the compound.
**DISCUSSION**

It is now generally recognised that novel compounds are called for treating leukaemic disease. This consideration prompted us to investigate the consequences of tetrahydroxyquinone on HL60 leukaemia cells, a compound most readily known as a primitive anti-cataract 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 anti-apoptotic survival molecules including the protein kinase B pathway in HL60 leukaemia cells but not their untransformed counterparts. Importantly, transfection of protein kinase B into HL60 cells and thus artificially increasing protein kinase B activity inhibited ROS-dependent cytotoxicity. We concluded that the remarkable cytotoxic effects tetrahydroxyquinone on HL60 leukaemia cells are dependent on reduced protein kinase B-dependent survival
signalling 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 signalling 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.

Figure 8. Schematic representation of tetrahydroxyquinone effects on HL60 leukaemia cells.

Tetrahydroxyquinone inhibits the anti-apoptotic PKB/Bad, NF-κB, and PKCδ signalling cassettes and stimulates the pro-apoptotic JNK cassette. Of these effects transfection studies reveal diminished activation of the anti-apoptotic PKB/Bad signalling cassette to be most important effect.

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 pro-inflammatory 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 signalling and activation of mitochondrial pathway of cell death, 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 present study we observed that ROS reduced the apparent activity of the NF-κB pathway, as deduced from the decrease in expression of both the ratio between p65 expression and I-κB as well as the reduced phosphorylation of IκB. As the NF-κB pathway is a potent survival signal, this down regulation corresponds well with the reduced survival signalling induced by ROS formation detected in this study. It is, however, more difficult to reconcile with the role of ROS generation in mediating inflammatory gene expression, as NF-kB is in general a strong pro-inflammatory transcription factor. Importantly, however, a recent study by Blanchette et al. [29] indicates that the contribution of NF-κB to macrophage activation-associated gene expression is minimal, hence our results are not necessarily at bay with the established role of ROS in the induction of inflammatory gene expression. Interestingly, increased JNK activity has recently come up as an important mediator of inflammatory gene expression in inflammatory bowel disease in vivo [30]. Hence, the ROS-induced activation of JNK may function in the induction of inflammatory gene expression.

Despite the intricacies of ROS action in cellular physiology, the present study has shown a remarkable action of tetrahydroxyquinone on HL60 leukaemia cells, acting through a novel survival signalling-inhibiting mechanism. Recent data indicate that impaired survival signalling through PKB/Akt has substantial clinical promise for the treatment of chemotherapy-resistant leukaemia [31]. Together, these observations make tetrahydroxyquinone a candidate drug for the treatment of leukaemia and further studies are under progress addressing its potential usefulness for combating disease.
REFERENCES


Chapter 2


