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Induction of CYP4F3 by benzene metabolites in human white blood cells *in vivo,* in human promyelocytic leukemic cell lines, and *ex vivo* in human blood neutrophils

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List of nonstandard abbreviations: LTB_4, leukotriene B_4; ATRA, *all-trans* retinoic acid; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; AML, acute myelogenous leukemia; CYP, cytochrome P450.

Abstract

Exposure to benzene elicits a spectrum of hematotoxicity ranging from reduction of peripheral blood cell counts to aplastic anemia and leukemia. The molecular mechanism by which benzene damages hematopoietic cells is unclear; in particular, benzene-induced aberrant gene expression has not been addressed. We analyzed differential gene expression in the peripheral white blood cells (WBC) from 7 female patients diagnosed with occupational benzene poisoning and 7 matched controls. Here, we report altered expression of cytochrome P450 (*CYP*) in the patients. All patients exhibited elevated expression of *CYP4F3A* encoding the leukotriene B4 (LTB$_4$) ω-hydroxylase critical in the inactivation of LTB$_4$ in polymorphonuclear leukocytes (PMN) with a fold of induction between 3 and 71. Four patients had high expression of *CYP1A1* and two patients had elevated expression of *CYP1B1*. Expressions of *CYP2B6*, *CYP51*, and *CYP27A1* were also altered in certain patients. Mechanistic analysis revealed that phenol, a major metabolite of benzene, significantly induced the expression of CYP4F3A at both mRNA and protein levels in cultured promyelocytic leukemia cells (HL-60) similarly to all-trans retinoic acid (ATRA). Induction of CYP4F3 by phenol was also observed in differentiated HL-60 cells, in pro-erythroid cell line K562, and *ex vivo* in human neutrophiles. On the other hand, hydroquinone induced extensive apoptosis of the cells. The findings demonstrated, for the first time, that benzene and metabolites induce CYP4F3 in human blood cells both *in vivo* and *in vitro*. Induction of CYP4F3 may play a role in the development of benzene hematotoxicity and serve as a biomarker of benzene exposure.

Introduction

Benzene is widely used in industries as a general purpose solvent and in the synthesis of other chemicals. Exposure to benzene occurs worldwide to workers in shoe making, automobile repair, oil industry, shipping, and chemical manufacture, and to the general population from cigarette smoking, gasoline vapor, and automobile exhaust (Wallace, 1996; ATSDR, 1997). Chronic exposure to benzene typically leads to bone marrow toxicity, which often initially manifests decreased peripheral blood cell counts (anemia, leukopenia, or thrombocytopenia) but may progress to pancytopenia, aplastic anemia, myelodysplasia, and ultimately myelogenous leukemia (AML) (Aksoy, 1989; Snyder et al., 1993b). The possibility that exposure to benzene at or below 1 ppm, which is the current occupational standard in US, may still cause toxicity to hematopoietic cells is an ongoing concern (Lan et al., 2004).

Benzene undergoes metabolic activation in the liver to form multiple metabolites that exhibit biological activities (Parke and Williams, 1953; Snyder and Hedli, 1996). Cytochrome P450 (CYP) 2E1, which metabolizes a large group of small molecular weight chemicals, plays a critical role in the initial and subsequent oxygenation of benzene giving rise to benzene oxide, phenol, hydroquinone, catechol, *E,E*-muconic acid, and other metabolites (Chepiga et al., 1991; Snyder et al., 1993a). Consistent with this notion, mice lacking CYP2E1 had relatively low levels of benzene metabolites and were resistant to benzene hematotoxicity compared with the wild type (Valentine et al., 1996). Other enzymes including microsomal epoxide hydroxylase (mEH, EPHX1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and glutathione-*S*-transferases (GSTs) participate in the formation of catechol, benzoquinone, and *S*-phenylmercapturic acid. In humans, polymorphisms of these enzymes were found to associate with the urine levels of the metabolites from benzene. Glutathione oxidoreductase (GSTT1) affects the production of *S*-phenylmercapturic acid and genetic polymorphisms in NQO1*2, CYP2E1, and EPHX1 influence the hepatic metabolism of benzene in workers exposed to benzene (Kim et al., 2007). Metabolites of benzene, such as phenol, hydroquinone, and muconaldehyde produced in the liver, may be transported to bone marrow via an as-yet-unclear mechanism to produce toxic effects in hematopoietic cells. Alternatively, metabolism of benzene and metabolites may occur in the marrow cells locally and

contribute to its toxicity. For instance, production of semiquinones and quinones and subsequently oxidative stress via myeloperoxidase in bone marrow cells has been proposed (Ross et al., 1996). It has been posited that benzene metabolites rather than benzene itself mediate the multiple biological effects of benzene on the hematopoietic cells to give rise to bone marrow toxicity.

The molecular targets of benzene metabolites and the signaling events governing the development of benzene-induced toxicity and cancer are not well understood. Protein and DNA adduct formation from benzene metabolites as well as oxidative stress due to the redox cycling potentials of quinone derivatives can potentially damage bone marrow cells. On the other hand, the chronic and progressive nature of benzene hematotoxicity and tumorigenecity suggests genomic reprogramming that would result in aberrant gene expression during the development of myelotoxicity and leukemia by benzene. However, few studies have been conducted to address this issue. We have utilized cDNA microarray to analyze gene expression profiles in peripheral white blood cells (WBC) from seven female workers who were diagnosed with occupational benzene poisoning in comparison with those from seven matched control subjects who did not have histories of occupational benzene exposure. The results revealed altered expression of a number of *CYP* genes. In particular, the expression of *CYP4F3A*, which encodes the leukotriene B_4 (LTB_4) ω-hydroxylase in WBC, was elevated in all patients with folds of increase from 3 to 71. Mechanistic analysis demonstrated that the benzene metabolite, phenol, but not benzene itself, induced CYP4F3 mRNA and protein expression in cultured promyelocytic leukemia cells (HL-60), pro-erythroid cells (K562), and *ex vivo* human neutrophiles. Induction correlated with increased production of PMN-stimulated superoxide anion. Hydroquinone, on the other hand, induced extensive apoptosis in the cells. The findings implicate induction of CYP4F3 in the development of benzene hematotoxicity and suggest it as a potential biomarker for occupational benzene poisoning.

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Methods

Human subjects. Seven female workers who had histories of occupational benzene exposure from a shoe manufacture factory and a chemical production company in the Xinxiang City, Henan Province, China, were diagnosed with varying degrees of benzene poisoning by the Xinxiang City Institute for Occupational Disease Control and Prevention (Table 1 & 2). Diagnosis was made according to the "Diagnostic Criteria of Occupational Benzene Poisoning" (GBZ-2002, Department of Health, China; mhtml:file://E:\benzene poisoning diagnosis standard.mht). Among the patients, 6 were diagnosed with "occupational chronic benzene poisoning" including 2 cases of moderate, 2 cases of medium, and 1 case of severe benzene poisoning, and 1 case of aplastic anemia; one patient was diagnosed as having "suspicious benzene poisoning". All patients exhibited reduction of peripheral blood cell counts; the severity of hematocytopenia paralleled with the degrees of benzene poisoning (Table 2). The concentrations of benzene that these patients were exposed to at the work place ranged from 10 to 210 mg/m^3 at the time of diagnosis. Seven local workers who did not have occupational benzene exposure histories and were matched with the patients on age (with variations of < 3 years of age), gender (female), geographical location, years of working experience, educational level, and history of smoking and alcoholic drinking, were chosen as controls (Table 1). The controls were shown to be healthy subjects upon physical examination (Table 2). Normal blood cell count values in adult Chinese are as follows (Wang, 2004): red blood cells: 5×10^{12}/L (male) and 4.2×10^{12}/L (female); hematocrit: 120~160 g/L (male) and 110~150 g/L (female); WBC: $4.0 \sim 10.0 \times 10^9$/L; neutrophil; 50~70%; eosinophil: 0.5~5%; basophil: 0~1%; lymphocyte: 20~40%; monocyte: 3~8%; and platelet: $100 \sim 300 \times 10^9$/L. No patients or controls had occupational histories of exposure to other hematotoxic chemicals or radiation and were not exposed to radiation therapy or chemotherapy within 15 days prior to the study. All procedures involving human subjects including information and sample collection, sample analysis, and informed consent were approved by the institutional review board of Wuhan University Medical Center and by the National Nature Science Foundation, China, and the Hubei Provincial Nature Science Foundation, Hubei, China.

Blood sample preparation and microarray analysis. Ten milliliters of peripheral blood were collected from the patients and controls. White blood cells were prepared from fresh blood samples using the Polymorphprep solution (AXIS-SHIELD, PoC AS, Oslo, Norway) following the instruction from the manufacturer. Total RNA was prepared using the Trizol reagents (Invitrogen, Carlsbad, CA, USA) per manufacturer's instruction. cDNA microarray was performed using the CSC-GE-80 microarray chip with cDNAs representing 8,064 human expressed genes, following the procedures provided by the chip manufacturer (Shenzhen Chipscreen Biosciences Ltd., Shenzhen, Guandong, China). Briefly, cDNA probes were prepared from total RNA samples from individual patients and matched controls by reverse transcription and were labeled with Cy3 (green, for the patient group) or Cy5 (red, for the control group), respectively. Seven chips and seven patient-control matched probes were denatured for 5 min at 95 ^{o}C, separately. The probes were applied to the surface of the chips. Hybridization was performed at 42 ^{o}C for 16 h, followed by washing once with 0.2x SSC containing 0.2% SDS and once with 0.2x SSC without SDS. The chips were scanned using the Generation III Array Scanner from Amersham Pharmacia (Piscataway, NJ, USA) to detect fluorescent signals. To ensure the quality of microassay, each chip contained a total of 384 genes as positive and negative controls, which assess the interference from non-specific hybridization signals, and internal standards (housekeeping genes), which measure the repeatability and stability of the results, as well as external standards, which measure the dose-response relationship and ratio quantification to reflect the reliability and sensitivity of the hybridization. Each control was repeated for 12 times on the same chip to assess variability of hybridization. A Cy3/Cy5 ratio of larger than 2 or less than 0.5 was considered significantly different between the patient and the control samples. Microarray data were analyzed using the software provided by Shenzhen Chipscreen Biosciences Ltd.

Cell culture and treatment. The HL-60 cell line derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia was obtained from American Type Culture Collection (ATCC, Manasus, VA, USA). The cells were cultured in the Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Calsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C and

5% CO_2. For a typical treatment, HL-60 cells were seeded in a 6-well plate at a density of $1x10^5$ cells/ml and were treated with benzene, phenol, and hydroquinone for 4 days at concentrations indicated in the figure legends. *All-trans*-retinoic acid (ATRA, 1 μM, 4 days) was used as a positive control. Benzene, phenol, and hydroquinone were dissolved in 70% ethanol and ATRA in dimethyl sulfoxide (DMSO). All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The pro-erythroid cell line, K562, was purchased from ATCC and cultured in IMDM with 10% FBS similarly to HL-60 cells.

Ex vivo induction in human blood neutrophiles. Whole blood from a healthy male adult was purchased from AllCells, LLC (Emeryville, CA, USA). Neutrophiles were isolated according a protocol provided by AllCells. Briefly, whole blood was diluted with a washing buffer (Dulbecco phosphate-buffered saline without calcium and magnesium but with 10% FBS and 2 mM EDTA). The Lymphocyte Separation Medium (StemCell Technologies, Inc., Vancouver, Canada) was laid on top. The tube was centrifuged at 400 xg for 20 min at 18 ^{o}C. The interface contains the lymphocyte fraction, whereas the bottom contains mostly red blood cells and neutrophiles. Red blood cells were lysed with a cold amomium chloride solution and the remaining cells (neutrophils) were washed for several times. Cell purity and viability were examined and were around 90-97% and 85-95%, respectively. The neutrophiles were cultured in IMDM with 10% FBS at 37 ^{o}C and 5% CO_2, and were treated with 100 μM or 1 mM phenol for 2 days. Total protein was prepared and analyzed by immunoblotting.

RNA measurement. Total RNA was prepared from cells using the Qiagen total RNA isolation kit (Qiagen, Valencia, CA, USA) and mRNA was reverse-transcribed into cDNA with RNA reverse transcriptase. The cDNA samples were analyzed by real-time PCR using the SYBR green PCR master mix from Applied Biosystems (Foster City, CA, USA) performed on a Bio-Rad iCycler (Bio-Rad) following standard procedures. Briefly, for each reaction, DNA template, forward and reverse primers (10 μM each), PCR master mix, and water were added to make a final volume of 50 μl. Thermal cycling was carried out as follows: 95°C for 3 min as initial denaturing, followed by 45 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, and a final extension at 72°C for 2 min. Threshold cycles (C_T

values) were determined using the iCycler IQ software (Bio-Rad). Relative DNA amounts were calculated from C_T values for each sample by interpolating into the standard curve obtained using a series of dilution of standard DNA samples run under the same conditions. The sequences of the primer sets used for real-time PCR are as follows. *CYP4F3*: forward, 5'-GAGGAGGTTGTGTGGGACAAGG; reverse, 5'-GTGGAAGATGCGGACGATTGCG. *CYP4F3B*: forward, 5'-TCCTCGCTGGGCCTTTGGCCAA; reverse, 5'-TGGGGTGGCAAAAACGGATGAC. To visualize results, real-time PCR was stopped at cycles at which differences between treatments and controls became apparent and the cDNAs amplified were then fractionated on a 1% agorase gel and were stained with ethadium bromide for detection under UV light.

Multiparameter flow cytometry for apoptosis. Apoptosis was analyzed with multiparameter flow cytometry as described previously (Telford et al., 2004; Hu et al., 2006). The assay measures three characteristic changes of apoptosis: caspase activation, plasma membrane phosphatidylserine (PS) "flipping", and increase in cell membrane permeability. PhiPhiLux-G1D2 (PhiPhiG1D2, OncoImmunin, Inc., Gaithersburg, MD, USA) is a fluorogenic substrate of caspase 3 and 7. 7-Aminoactinomycin D (7-AAD, BD Bioscience, San Diego, CA, USA) is used as an indicator of plasma membrane permeability. Annexin V-PE (BD Bioscience) binds to "flipped" PS of plasma membrane. Briefly, HL-60 cells were treated for 4 days as indicated in figure legends. Cadmium (10 or 40 µM, 16 h) was used as a positive control for apoptosis. Cells were collected by centrifugation at 1,500 rpm for 5 min. The supernatant was re-centrifuged at 2,500 rpm for 5 min to collect apoptotic cells that were not collected during the 1st centrifugation. The cell pellets were combined and re-suspended in the medium at a density of ~ $3x10^6$ cells/ml. Fifty microliters of the cell suspension were mixed with 50 µl of 10 µM PhiPhiG1D2, followed by incubation at 37^oC for 60 min. Incubation was continued with 1.25 µl of annexin V-PE at room temperature for 15 min. The cells were washed with 1 ml of ice-cold FACS buffer (0.2% BSA and 0.09% NaN_3 in phosphate-buffered saline), collected by centrifugation at 800 xg for 5 min, and re-suspended in 250 µl of the FACS buffer. The cells were incubated with 7-AAD (0.06 mg/1.25 ml/tube) at room

temperature for 10 min. Two hundred microliters of the FACS buffer were added to the tube immediately before measurement with a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA).

Detection of CYP4F3 protein. Cells were treated as indicated in figure legends. For immuno fluorescent labeling and flow cytometry of the CYP4F3 protein, the cells were labeled with polyclonal anti-CYP4F3 antibodies (Abnova, Taipei, Taiwan) followed by fluorescent labeled 2nd antibodies. Fluorescent signals were measured as described above. For immunoblotting, total cell extracts were fractionated in SDS-polyacrylamide gel, transferred to a nitran membrane, and stained with the anti-CYP4F3 antibodies followed by horse radish peroxidase-conjugated secondary antibodies and chemiluminescent detection. Quantification of protein bands was performed with the ImageQuant program (Molecular Dynamics, San Jose, CA, USA).

NBT reduction assay. HL-60 cells stimulated with PMA (phorbol myristate acetate) generate superoxide anion; production of this anion was measured by reduction of NBT (nitroblue tetrazolium) to form intracellular reduced blue-black formazen deposits (Collins et al., 1979). Briefly, cells were seeded at a density of $1x10^5$ cells/ml in 6 well plates. Three untreated, three treated with 0.1% DMSO, and three with 0.1% ethanol were used as negative controls. ATRA at 1 µM was used as a positive control. The cells were treated with benzene, phenol, or hydroquinone as indicated. After 6 days of treatment, the cells were collected by a centrifugation at 1000 rpm for 5 min. Cell pellets were re-suspended with 1 ml of a freshly prepared mixture containing 0.1% NBT and 1 µg/ml of PMA in the medium and were incubated at 37^oC for 1 h. Slides containing cell smears were stained with May-Grunwald-Giemsa (Sigma-Aldrich). The percentage of cells containing intracellular dark blue-black formazen deposits were counted under a light microscope at 40x magnification for at least 200 cells per sample.

Benzidine staining. Cell hemoglobinization was analyzed by benzidine staining (Belhacene et al., 1998). K562 cells (ATCC) were cultured in IMDM with 10% FBS and 5% CO_2. Fifty microliters of cells at $1x10^6$ cells/ml was mixed with 10 µl of a benzidine reagent consisting of 0.6% H_2O_2, 0.5 mol/L acetic acid, and 0.2% benzidine dihydrochloride (Sigma). The percentage of benzidine positive cells

(blue cells) was determined by light microscopic examination of 100 cells per sample. Each experiment was performed in triplicate and the results were averaged.

Statistical analysis. Means and standard deviations (SDs) were calculated, and statistical analyses were performed using one-way ANOVA followed by *t*-test, by using the GraphPad PRISM program (GraphPad Software, Inc., San Diego, CA, USA).

Results

Elevated expression of CYP4F3A in the peripheral WBC from patients diagnosed with occupational benzene poisoning. To analyze aberrant gene expression and its role in the development of benzene hematotoxicity, we carried out cDNA microarray to compare the gene expression profiles of peripheral WBC from occupational benzene poisoning patients and matched controls. The patient group included seven female workers with occupational benzene exposure histories from 7 to 20 years while working in a shoe manufacture factory or a chemical production company. The workers were diagnosed with varying degrees of occupational benzene poisoning (Table 1 & 2), including "suspicious benzene poisoning" (1 case), moderate chronic benzene poisoning (2 cases), medium chronic benzene poisoning (2 cases), severe chronic benzene poisoning (1 case), and aplastic anemia (1 case) (Table 2). The WBC, neutrophil, lymphocyte, and platelet counts and hematocrit of the patients at the time of blood sampling or diagnosis were shown in Table 2. All patients showed reduction of WBC counts at the time of diagnosis and blood collection. The patients with aplastic anemia and severe level benzene-poisoning also exhibited anemia and thrombocytopenia. The control subjects consisted of seven local healthy female workers, who did not have occupational benzene exposure histories but were matched with the patients for age, geographical location, years of work, educational level, and histories of smoking and alcoholic drinking. The blood cell counts of the matched controls were shown in Table 2.

The results from matched cDNA microarray revealed altered expressions of multiple genes that are involved in apoptosis, DNA repair, immune function, and drug metabolism in the peripheral WBC of patients with benzene poisoning compared with those of healthy controls. Since metabolism plays an important role in benzene toxicity, we analyzed the expression profiles of *CYP*s in this study. We found that the expression of *CYP4F3A,* which encodes LTB_4 ω-hydroxylase, was elevated in all patients (Table 3). The fold of induction varied from 3 to 71 with a geometric mean of 9-fold (Table 4). Induction of *CYP4F3A* was also confirmed by real-time PCR (data not shown). Induction of the *CYP4F3A* mRNA did not appear to correlate with the severity of hematotoxicity in the patients in both microarray and real-time PCR assays. *CYP1A1* and *1B1*, two polycyclic aromatic hydrocarbon (PAH)-inducible forms of *CYP*s,

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were elevated in 4 and 2 patients, respectively. *CYP2B6* and *CYP51* were down regulated in one patient, whereas *CYP27A1* was up-regulated in one patient but was down-regulated in 3 patients. CYP4F3A catalyzes the conversion of LTB$_4$, a potent endogenous chemotactic and chemokinetic signaling molecule, to less active products in PMN cells. Induction of CYP4F3A enhances the inactivation of LTB$_4$. The consistent elevation of CYP4F3A in all benzene poisoning patients suggests a regulatory mechanism by which benzene or its metabolites induce the gene in WBC, which may contribute to the pathogenesis of hematotoxicity in humans.

Induction of the CYP4F3 subfamily in HL-60 cells. The findings that *CYP4F3A* expression was significantly elevated in all patients and yet the fold of induction did not appear to correlate with the severity of the diagnosis suggest that induction is a common feature of benzene poisoning rather than a result of hematocytopenia. Moreover, since *CYP4F3A* is mostly expressed in neutrophils and yet all patients had normal or reduced differential neutrophil counts, it is unlikely that induction of *CYP4F3A* was a result of an increase in the relative amounts of neutrophils in the blood, but rather was due to increased expression of the gene. To further examine this posit, we analyzed the effect of benzene and its metabolites on *CYP4F3A* gene expression in HL-60 cells, a cell line derived from human acute promyelocytic leukemia; this cell line has previously been shown to exhibit elevated expression of *CYP4F3A* upon exposure to chemicals such as ATRA (Kikuta et al., 2004). A time-course study of *CYP4F3A* mRNA induction indicated a slow process requiring longer than 2 days of exposure for apparent induction (data not shown). Both *CYP4F3A* and *CYP4F3B*–a splice variant of *CYP4F3A*– mRNAs were expressed in HL-60 cells at low but detectable levels by RT-PCR (Figure 1A, lane 1). ATRA, which is known to induce *CYP4F3A* in HL-60 cells, was used as a positive control. ATRA at 1 µM with a 4 day treatment induced the mRNA expression of *CYP4F3A* significantly, and *CYP4F3B* to a much lesser extent (Figure 1A, lane 2). Benzene at a high concentration (10 mM, 4 days) only modestly induced *CYP4F3A* (lane 4), whereas phenol at 1 mM and 100 µM (4 days) induced both genes significantly (lanes 5 & 6). Treatment with hydroquinone at 100 µM for 4 days dramatically reduced the

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cell number of HL-60 cells yielding insufficient amount of mRNA for the analysis of *CYP4F3* expression (See data below). Quantification of *CYP4F3A* and *4F3B* mRNA expression by real-time quantitative PCR revealed that benzene at 10 mM induced *CYP4F3* by 2-fold; phenol at 1 mM and 100 µM induced *4F3* by 4 and 3.5-fold, comparable to the induction by ATRA (~5 fold) (Figure 1B). In addition, phenol at both concentrations induced *4F3B* by ~9 and 6-fold, respectively, but ATRA induced *4F3B* only slightly (~2-fold) (Figure 1C). Benzene had no apparent inductive effect on *4F3B* mRNA expression.

Induction of the CYP4F3 protein in HL-60 cells was performed using immuno fluorescent flow cytometry. ATRA induced the CYP4F3 protein at 1 µM as expected. Phenol induced CYP4F3 protein in a concentration-dependent manner at 10 and 100 µM, but induction was reduced at higher concentrations of phenol (10 mM) (Figure 2A). Induction by phenol was also time-dependent with high inductions between 4 and 6 days of treatment (Figure 2B). Immunoblotting confirmed that ATRA and phenol indeed induced the CYP4F3 protein that was recognized by the anti-4F3 antibodies as a protein band of ~55 kDa (Figure 2C&D). CYP4F3 proteins were nearly undetectable in untreated HL-60 cells by immunoblotting. Induction is ~5 fold for both inducers. The findings suggest that the benzene metabolite, phenol, induced CYP4F3A at both mRNA and protein levels in human leukocytes during benzene poisoning.

Induction of CYP4F3 correlates with induction of differentiation but not apoptosis by benzene metabolites. Expression of CYP4F3 in peripheral leukocytes can be influenced by the differentiation and functional status of the cells. Therefore, the effects of benzene and its metabolites on the growth and differentiation of HL-60 cells were analyzed in order to elucidate the mechanism of CYP4F3 induction by benzene. ATRA affected HL-60 growth only after 4 days of treatment suggesting a slow effect of ATRA on HL-60 growth (Figures 3&4). Benzene and phenol at high concentrations (10 and 1 mM, respectively) had only modest effects on the growth of the cells. On the other hand, hydroquinone at 100 µM induced a dramatic reduction in the cell numbers after one day treatment, which continued to decrease throughout the treatment resulting in >85% reduction in cell number after 4 days of treatment (Figures 3&4).

The dramatic and progressive reduction in cell numbers by hydroquinone can be attributed to inhibition of cell growth or induction of apoptosis and/or necrosis in HL-60 cells. The apoptotic effect of hydroquinone on the cells was examined using a multiparameter flow cytometric approach. Treatment with hydroquinone at 100 µM and 1 mM for 4 days caused apoptosis in ~75 and 70% of the cells, respectively, which was comparable to the apoptotic effects of treatment with cadmium at 10 or 40 µM for 16 h (positive controls for apoptosis) (Figure 5). Treatment with ATRA (1 µM), benzene (1 and 10 mM), or phenol (100 µM and 1 mM) for 4 days did not cause significant increases in apoptosis compared with the control. Thus, hydroquinone, but not benzene or phenol, caused apparent apoptosis in HL-60 cells.

Induction of differentiation of HL-60 cells by benzene and metabolites was analyzed by measuring PMA-stimulated production of superoxide anion, which reduces NBT to insoluble colored formazen deposits in cells quantitatively. As expected, upon treatment with ATRA (1 µM, 6 days; positive control for differentiation), 94.3% of the HL-60 cells were converted to NBT-positive cells, indicating induction of differentiation of most cells toward mature mononucleocytes or PMN (Figure 6A&B). Benzene at 10 mM caused a slight increase in NBT-positive cells from 13% (control) to 32% (Benzene), whereas phenol at 1 mM caused a significant increase to 65%. On the other hand, cells treated with hydroquinone at 100 µM and 1 mM all appeared apoptotic with small and shrunk nuclei as revealed under a light microscope. Figure 6C showed the morphology of untreated and ATRA or phenol-treated HL-60 cells, indicating that both ATRA and phenol induced differentiation of HL-60 cells from promyelocytic cells into mononucleocyte and PMN-like cells; consistent with this observation, no basophils or eosinophils were found with or without ATRA or phenol. The findings revealed that benzene induces the *CYP4F3* subfamily through its metabolite, phenol, and that induction of the *CYP* genes correlates with the ability of benzene metabolites to induce differentiation of HL-60 cells.

Ex vivo induction in human blood neutrophils. The correlation between induction of *CYP4F3A* in HL-60 and differentiation of the cells by phenol raised the possibility of whether phenol

induces the gene indirectly through induction of HL-60 differentiation. To test the possibility, we measured induction in differentiated HL-60 cells. The cells were treated with 1.3% DMSO for 4 days to induce differentiation, followed by treatment with phenol at 100 µM for additional 4 days. Whereas CYP4F3 was nearly undetectable in undifferentiated HL-60 cells by immunoblotting (Figure 2C&D), the protein level was elevated in differentiated cells induced by 1.3% DMSO; thus, differentiation indeed induces CYP4F3 protein expression (lane 1, Figure 7A&B). Phenol at 100 µM further increased CYP4F3 protein expression (lane 2, Figure 7A&B). The result supports the notion that phenol is capable of inducing CYP4F3 independently of differentiation in HL-60 cells.

We also examined if phenol induces differentiation and CYP4F3 induction in K562, an erythroleukemic cell line. Whereas hemin, a known inducer of K562 for differentiation, induced K562 into erythroid cells as indicated by induced synthesis of hemoglobin, phenol did not affect the amount of hemoglobin or induce morphology changes in K562 cells as compared with untreated controls (Figure 8A and data not shown). However, under the same conditions, phenol but not hermin induced CYP4F3 protein expression (Figure 8B&C).

Finally, we performed *ex vivo* induction of CYP4F3 in human blood neutrophil cells. Human neutrophils were isolated from blood from a healthy adult male and were treated with phenol at 100 µM and 1 mM in culture for 2 days (the life span of PMN in peripheral blood is ~2-3 days). CYP4F3 proteins were low in untreated cells; phenol treatment at both concentrations for 2 days induced the proteins expression by nearly 6-fold (Figure 9A&B). The *ex vivo* findings provided direct evidence demonstrating that phenol induces CYP4F3 in human neutrophils and induction does not require cell differentiation.

Discussion

Chronic exposure to benzene characteristically causes hematotoxicity and myelogenous leukemia in humans. Given the high specificity and a chronic and progressive course of damage to bone marrow cells by benzene, we posit that exposure to benzene elicits genomic reprogramming to result in a large scale aberrant gene expression in hematopoietic cells that in turn contributes to the development of hematotoxicity and leukemia. Indeed, microarray analysis of the gene expression profiles in the peripheral WBC from patients diagnosed with occupational benzene poisoning in comparison with those of healthy controls have uncovered altered expressions of a broad range of genes. Aberrant gene expressions were found in a number of pathways that have been implicated in hematopoietic functions and cancer, including DNA damage repair, immune function, apoptosis, and drug metabolism (Chen et al., 2005; Xia et al., 2005; Zhao et al., 2005). Recently, a separate group of researchers reported altered expressions of a set of genes, in particular *CXCL16*, *ZNF331*, *JUN*, and *PF4*, in the peripheral mononucleocytes from workers who were exposed to benzenes (but not diagnosed with benzene poisoning), compared with controls who did not have histories of occupational benzene exposure (Forrest et al., 2005). It was suggested that these genes can potentially be used as biomarkers for benzene exposure in humans. Taken together, these findings underpin the potential role of gene regulation by benzene in benzene-induced hematoxicity and leukemia.

Although benzene metabolism by CYPs has long been recognized as required for benzene toxicity and was extensively studied in various systems including reconstituted P450 reactions, cultured cells, whole animals, or benzene-exposed human populations (Parke and Williams, 1953; Snyder, 2002; Kim et al., 2007), few studies have been conducted to examine the effects of benzene exposure on the expression of CYPs and whether these effects contribute to benzene hematotoxicity. To address this issue, we focused on the analysis of altered expression of CYPs in workers diagnosed with chronic occupational benzene poisoning using cDNA microarray. The data revealed differential expressions of a number of *CYP* genes, including *CYP*s *4F3A*, *1A1*, *1B1*, *27A1*, *2B6*, and *51*, in the peripheral white blood cells of the patients in comparison with matched controls. In particular, the expression of *CYP4F3A* was

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consistently elevated in all patients with folds of induction from 3 to 71. Since these *CYP*s participate in the metabolism of a broad range of endogenous and foreign chemicals including benzene, the results suggest that exposure to benzene affects the metabolism of its own as well as many other endogenous substrates or co-exposed toxicants by way of modulating the expression of *CYP*s, which potentially contributes to the development of benzene hematotoxicity.

CYP4F3A encodes the LTB_4 ω-hydroxylase in human peripheral leukocytes, mostly PMNs that converts LTB_4, a potent and critical chemotactic agent for PMN, to less active molecules (Samuelsson, 1983; Shak and Goldstein, 1984; Kikuta et al., 1993; Christmas et al., 1999). Induction of the enzyme would increase the inactivation of LTB_4 and consequently inhibits the chemotaxis and infiltration of PMN and macrophages into tissues during inflammation. In the case of benzene-induced hematotoxicity, induction of CYP4F3 in peripheral white blood cells potentially contribute to the pathological development in two ways. Induction of CYP4F3 reduces chemotaxis and the migration of white blood cells from the circulating blood into tissues, resulting in an inhibition of inflammatory responses and consequently, contributing to increased susceptibility to microbe infections–a complication commonly seen in benzene hematotoxicity patients, although leucopenia may be the main cause of increased infections in the patients. Secondly, reduced tissue infiltration of blood cells would extend the lifespan and promote the proliferation of benzene-transformed cells or leukemia cells within blood vessels. Further studies are needed to support these notions.

Several potential mechanisms may account for the induction of *CYP4F3A* in the WBC of benzene poisoning patients. First, induction can be a result of neutrophile expansion that may occur in benzene-poisoning patients; in this scenario, an increase in *CYP4F3A* in the peripheral blood cells reflects increased relative amount of neutrophiles that express *CYP4F3A*. However, the fact that all patients have normal or reduced neutrophile counts negates this possibility. Second, induction can be secondary to hematocytopenia in the patients. This is unlikely, since *CYP4F3A* expression was elevated in all patients but the fold of induction did not correlate with the severity of leukopenia of the patients, indicating that reduction of blood cells per se was not sufficient for inducing the gene. Third, induction is a direct result

of exposure to benzene and/or its metabolites. *In vitro* induction of CYP4F3 at both mRNA and protein levels by the benzene metabolite, phenol, in cultured human promyelocytic leukemia cells, similarly to ATRA–a known *CYP4F3A* inducer, strongly supports this notion. Moreover, induction was specific in that only phenol but not benzene or hydroquinone induced the gene. The observation that phenol induces CYP4F3 protein expression in differentiated HL-60 cells and in K562 cells indicated that induction is independently of cell differentiation. Finally, our *ex vivo* experiment with human blood neutrophils provided direct evidence demonstrating that phenol induces CYP4F3 protein expression in neutrophils. To our knowledge, this is the first evidence of induction of CYP4F3 in human blood cells by chemicals.

The molecular target of phenol for the induction of the *CYP4F3* subfamily remains to be identified. The observation that phenol induced both *4F3A* and *4F3B*, whereas ATRA induced only *4F3A*, suggests that induction by the two chemicals may be mediated via overlapping but not identical pathways. *CYP4F3A* is normally expressed in PMN cells, whereas *CYP4F3B* is believed to be mainly detected in the liver as a splice variant of *CYP4F3A* (Christmas et al., 1999). The finding that both *CYP4F3A* and *4F3B* are induced by phenol in cultured HL-60 cells indicates that the two members of the *CYP4F3* subfamily share a common mechanism of regulation in HL-60 cells despite the preferential expression of *4F3A* in WBC and *4F3B* in the liver *in vivo*.

In contrast to phenol, hydroquinone induced rapid and extensive apoptosis of the cells. Since the reduction of cell counts in both bone marrow and peripheral blood is a hallmark of clinical signs in the early stages of benzene hematotoxicity, the results suggest that hydroquinone contributes significantly to the development of benzene myelotoxicity, in particular leukocytopenia. Taken together, these findings are consistent with the notion that multiple metabolites of benzene mediate the diverse biological effects of benzene in bone marrow and peripheral blood cells leading to progressive hematotoxicity and leukemia.

The widespread exposure to benzene worldwide from a broad range of occupational and environmental sources and the possibility that low dose exposure of benzene (at or <1 ppm) can still damage bone marrow cells raise health concerns for benzene-exposed workers as well as the general

population. Moreover, due to the lack of knowledge on the mechanism of benzene action on hematopoietic cells at molecular levels, mechanism-based measures for the prevention and treatment of benzene hematotoxicity is lacking. Our findings of large-scale alterations of gene expression in the peripheral white blood cells of workers with chronic benzene poisoning provide not only new mechanistic insights into the pathogenesis of benzene hematotoxicity and leukemia, but also opportunities for developing novel strategies for the diagnosis, prevention, and therapy of benzene-induced bone marrow diseases. In this regard, the consistent elevation of *CYP4F3A* in WBC of benzene poisoning patients suggests that it may be used as a biomarker for benzene exposure and benzene hematotoxicity in human populations in the future.

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Footnotes

*Z.Z. and X.H. made equal contributions to the paper.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Figure Legends

Figure 1   Induction of *CYP4F3A* and *4F3B* mRNA. HL-60 cells were cultured in IMDM with 10% FBS in suspension at a density of 1×10^5 cells/ml and were treated with ATRA, benzene, phenol, or hydroquinone as indicated for 4 days. Total RNA was prepared. *CYP4F3A* and *4F3B* mRNA expression was examined using RT-PCR and agarose gel electrophoresis (A) or using real-time quantitative PCR (B & C). Data represent means and standard deviations from 3 experiments. Primers used for PCR were described under Material and Methods.

Figure 2   Induction of CYP4F3 protein. HL-60 cells were treated as indicated. The CYP4F3 protein was detected and quantified using immuno fluorescent flow cytometry or immunoblotting with antibodies against CYP4F3. (A) Induction of CYP4F3 protein by phenol. Cells were treated for 4 days. ATRA at 1 µM was used as a positive control. (B) Time curve of CYP4F3 protein induction by phenol (100 µM). (C) Immunoblotting. (D) Quantification of immunoblotting results. Fluorescent flow cytometry was performed as described under Methods. For immunoblotting, total cell lysates of HL-60 cells treated with ATRA or phenol for 5 days were fractionated on SDS-PAGE (30 µg protein/lane). CYP4F3 protein was detected with the anti-4F3 antibodies. Actin was blotted as loading control.

Figure 3   Effect of benzene and metabolites on cell growth. HL-60 cells were treated as indicated for 4 days. Cell growth was assessed by counting cell numbers. Data represent means and standard deviations from 4 samples.

Figure 4   Time curve of inhibition of cell growth. HL-60 cells were treated with ATRA, benzene, phenol, or hydroquinone for indicated time periods. Cell growth was assessed by counting cell numbers. The cell densities were normalized with the controls (data not shown). Data represent means and standard deviation s from 4 samples.

Figure 5   Apoptosis induced by benzene and metabolites in HL-60 cells. Cells seeded at a density of 1×10^5 cells/ml were treated with ATRA, benzene, phenol, or hydroquinone as indicated for 4 days. Treatments with cadmium 10 µM and 40 µM for 16 h were used as positive controls for apoptosis.

Apoptosis was analyzed by using multi-parameter flow cytometry as described under Methods. Cells stained positive for annexin V and PhiPhiG1D2 were counted as apoptotic cells. Data represent means and standard deviations from three samples.

Figure 6   HL-60 cells were treated with ATRA (1 µM), benzene (1 and 10 mM), phenol (0.1 and 1 mM), or hydroquinone (0.1 and 1 mM), and were cultured for 6 days. The cells were incubated with 0.1% NBT and 1 µg of TPA for 1 h at 37°C. The percentage of dark blue-black (NBT positive) cells was counted under a light microscope for at least 200 cells per sample. The percentage of NBT-positives in untreated cells was not statistically different from those of cells treated with 0.1% DMSO or 0.1% ethanol (solvent controls). (A) Micrographs of representative HL-60 cells under different treatments stained with May-Grunwald-Giemsa showing dark blue-black intracellular deposits of formazen. (B) Quantitation of NBT-positive cells (% of counted cells) from five view fields for each treatment. Scale bar = 50 µm. (C) Morphology of HL-60 cell differentiation. Cells were stained with Wright's Giemsa and were examined under light microscope (magnification = 100X). Control cells (undifferentiated) were round shaped with a uniform cell size, large circular nuclei, but little cytoplasm. Treatments with ATRA or phenol induced differentiation of HL-60 cells into mononucleocyte and neutraphile-like cells with variable cell sizes, increased cytoplasm, condensed nuclei, and polymorphic nuclei.

Figure 7   Differentiation and induction in HL-60 cells. HL60 cells were treated with 1.3% DMSO for 4 days to induce differentiation and the cells were continued to be cultured with or without phenol (100 µM) for additional 4 days. Total protein was prepared and analyzed by immunoblotting with anti-4F3 antibodies. Actin was blotted as a loading control. (A) Immunoblotting. (B) Quantification of immunoblotting results.

Figure 8   Induction of differentiation and CYP4F3 expression in K562 cells. (A) Effect of phenol on K562 differentiation. K562 cells were treated with phenol (1 mM) or hemin (30 µM, positive control) for 96 h. Cell hemoglobinization representing differentiation into erythroid cells was analyzed using benzidine staining. Data represent percent of cells positive for benzidine staining (mean ± standard

deviation). ** Indicate $p<0.01$. (B) Induction of CYP4F3. K562 cells were treated as described for (A).

Total cell lysates were analyzed by immunoblotting for CYP4F3 expression. Actin was blotted as

loading control. (C) Quantification of immunoblotting results.

     Figure 9 *Ex vivo* induction of CYP4F3. Human blood neutrophils were treated with phenol at

100 μM and 1 mM for 2 days. (A) Total cell lysates were immunoblotted for CYP4F3 protein

expression. Actin was blotted as loading control. (B) Quantification of immunoblotting results.

Table 1 Age and Benzene-Exposure Time of Chronic Benzene Poisoning Workers and Age and Working Duration of Matched Controls

Patient Number	Benzene Poisoning Workers		Matched Conrols	
	Age (years)	Exposure Time (Years)	Age (years)	Working Duration (years)
1	36	7	35	7
2	45	14	46	13
3	40	20	40	19
4	47	12	46	13
5	42	7	44	8
6	35	13	36	12
7	55	18	56	20

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Table 2. Diagnosis and Peripheral Blood Cell Counts[a]

Patient Number	Diagnosis of Chronic Benzene-Poisoning	WBC 10^9/L	Neutrophil %		Lymphocyte %		Hematocrit g/L		Platelet 10^9/L	
Patient Group										
1	Suspicious	4.6 (3.05)[b]	69	(62)	31	(38)	125	(112)	170	(120)
2	Moderate Level	3.5 (3.2)	51	(48)	49	(51)	122	(108)	190	(170)
3	Aplastic Anemia	2.9 (2.0)	34.5	(32)	52.8	(54)	108	(62)	51	(32)
4	Medium Level	2.6 (2.2)	52	(53)	48	(47)	110	(110)	140	(130)
5	Medium Level	2.2 (2.4)	60	(57)	40	(43)	112	(125)	120	(116)
6	Severe Level	3.7 (2.8)	59.2	(58)	33.6	(32)	90	(84)	90	(82)
7	Moderate Level	3.2 (3.1)	65	(62)	35	(38)	120	(104)	210	(140)
Control Group										
1	-	8.6	62		38		138		230	
2	-	6.9	56		43		134		180	
3	-	6.9	58		42		118		170	
4	-	5.6	52		48		129		210	
5	-	8.2	61		39		130		160	
6	-	5.3	59		41		126		220	
7	-	8.6	60		40		132		140	

[a] Diagnosis was made according to the "Diagnostic Criteria of Occupational Benzene Poisoning" (GBZ-2002, Department of Health, China at website mhtml:file://E:\benzene poisoning diagnosis standard.mht). Normal blood cell count values in adult Chinese: (Wang, 2004): red blood cells: 5×10^{12}/L (male) and 4.2×10^{12}/L (female); hematocrit: 120~160 g/L (male) and 110~150 g/L (female); WBC: 4.0~10.0×10^9/L; neutrophil; 50~70%; eosinophil: 0.5~5%; basophil: 0~1%; lymphocyte: 20~40%; monocyte: 3~8%; and platelet: 100~300×10^9/L.

[b] Numbers represent peripheral blood cell counts at the time of sample collection or diagnosis (in parenthesis).

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Table 3. Differential Expression of CYP Genes in Chronic Benzene Poisoning Workers and Matched Controls

GenBank	GeneTag	Gene Function	Up-Regulation	Down-Regulation
A1927693	CYP4F3A	CYP450, subfamily IVF, polypeptide 3 (leukotriene B4 Omega hydroxylase)	7/7	
AA418907	CYP1A1	CYP450, subfamily I (aromatic compound-inducible), Polypeptide 1	4/7 (2,5,6,7)[a]	
N66957	CYP27A1	CYP450, subfamily XXVIIA (steroid 27-hydroxylase, cerebrotendinous xanthomatosis), polypeptide 1	1/7 (1)	3/7 (2,5,6)
AA448157	CYP1B1	CYP450, subfamily I (dioxin-inducible), polypeptide 1 (glaucoma 3, primary infantile)	2/7 (4,6)	
T68287	CYP2B6	CYP450, subfamily IIB (Phenobarbital-inducible), Polypeptide 6		1/7 (1)
AA477781	CYP51	CYP450, 51 (lanosterol 14-alpha-demethylase)		1/7 (1)

[a] Numbers in parenthesis refer to the benzene-poisoning patient numbers.

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Table 4. Fold of Induction of CYP4F3A mRNA Expression in Chronic Benzene-Poisoning Patients Relative to Matched Controls

Patient Number	Diagnosis	Fold of Up-Regulation
1	Suspicious Benzene Poisoning	2.96
2	Moderate Level Benzene Poisoning	11.8
3	Aplastic Anemia	3.92
4	Medium Level Benzene Poisoning	22.2
5	Medium Level Benzene Poisoning	3.94
6	Severe Level Benzene Poisoning	2.96
7	Moderate Level Benzene Poisoning	70.6
Average Fold of Induction		9.012 (1.22)[a]

[a] Represents geometric mean (standard deviation).
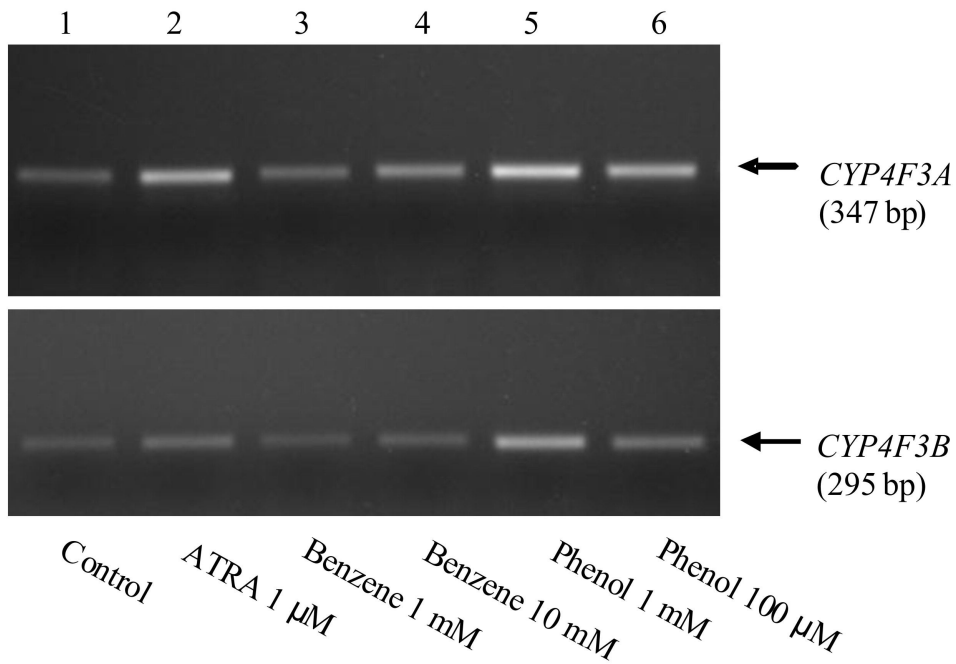
Figure 1A
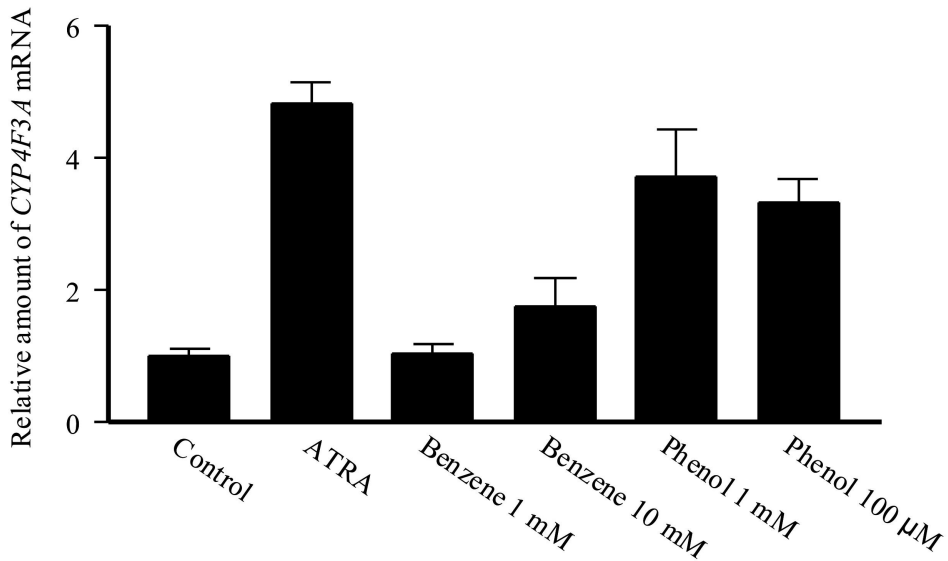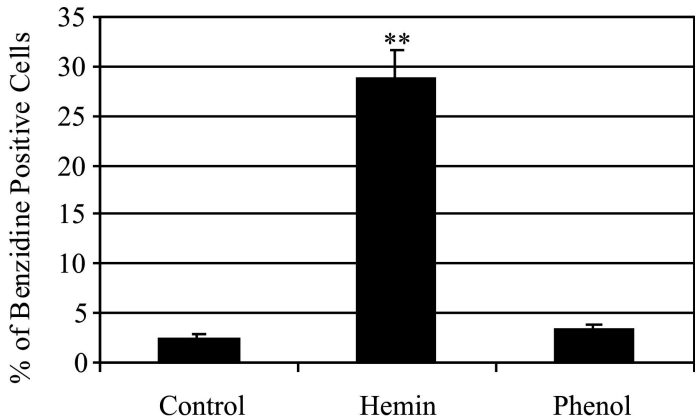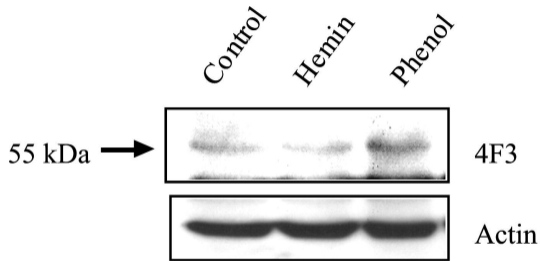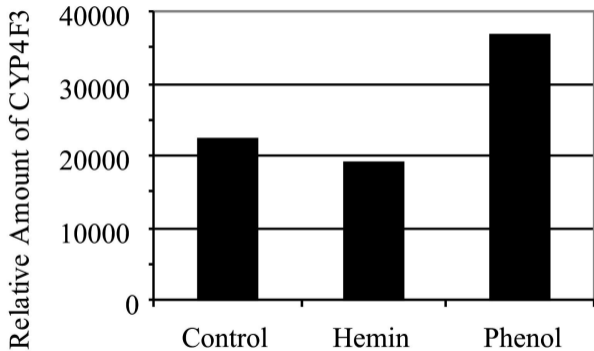
Figure 1B
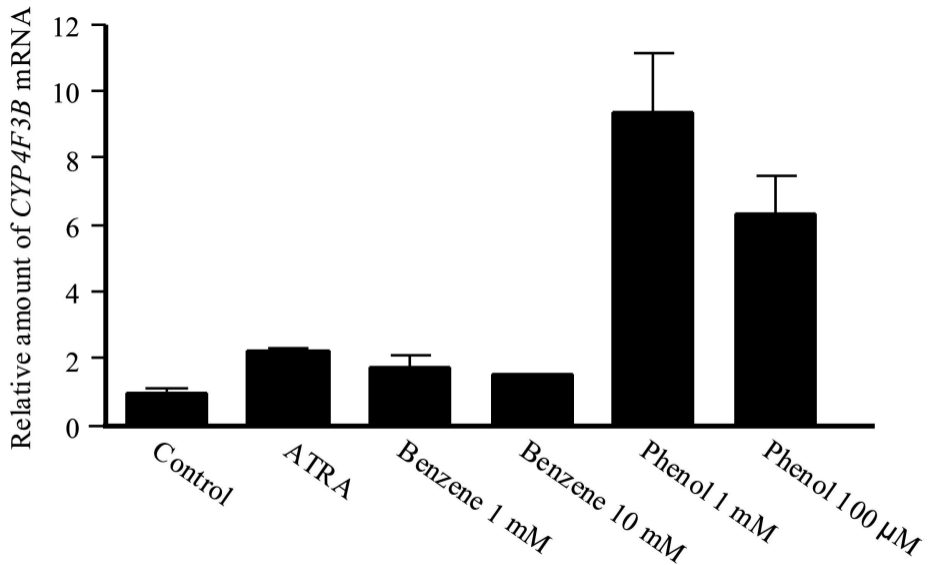
Figure 1C

Figure 2A

Figure 2B

Control   ATRA   Phenol

55 kDa ➤   4F3

Actin
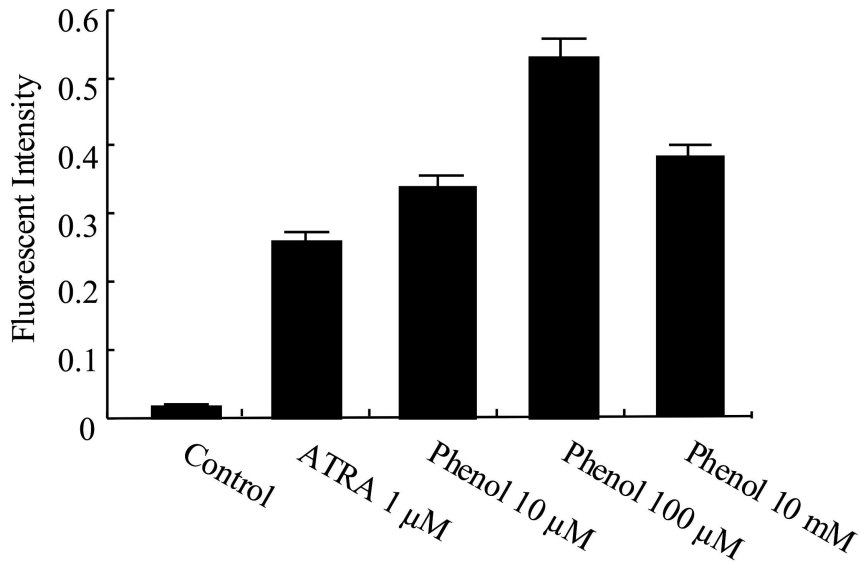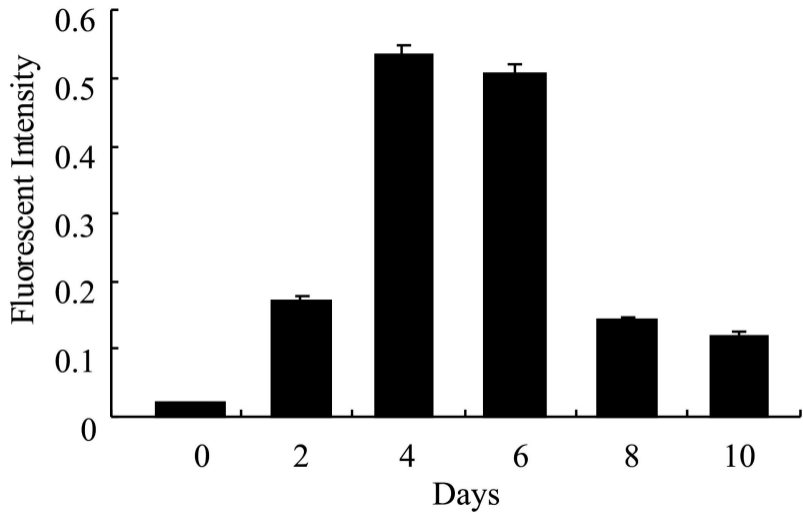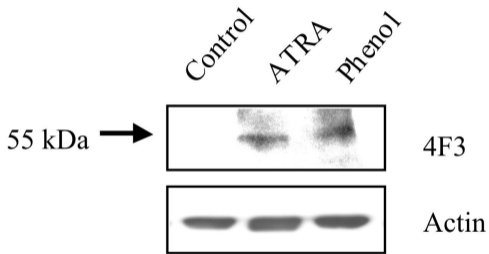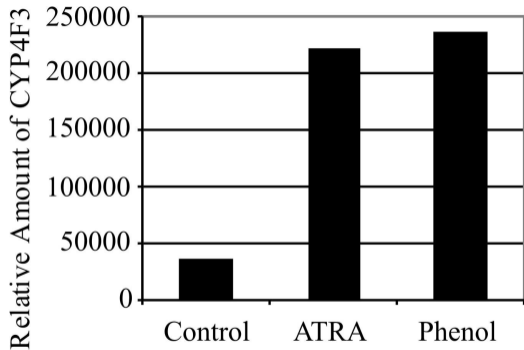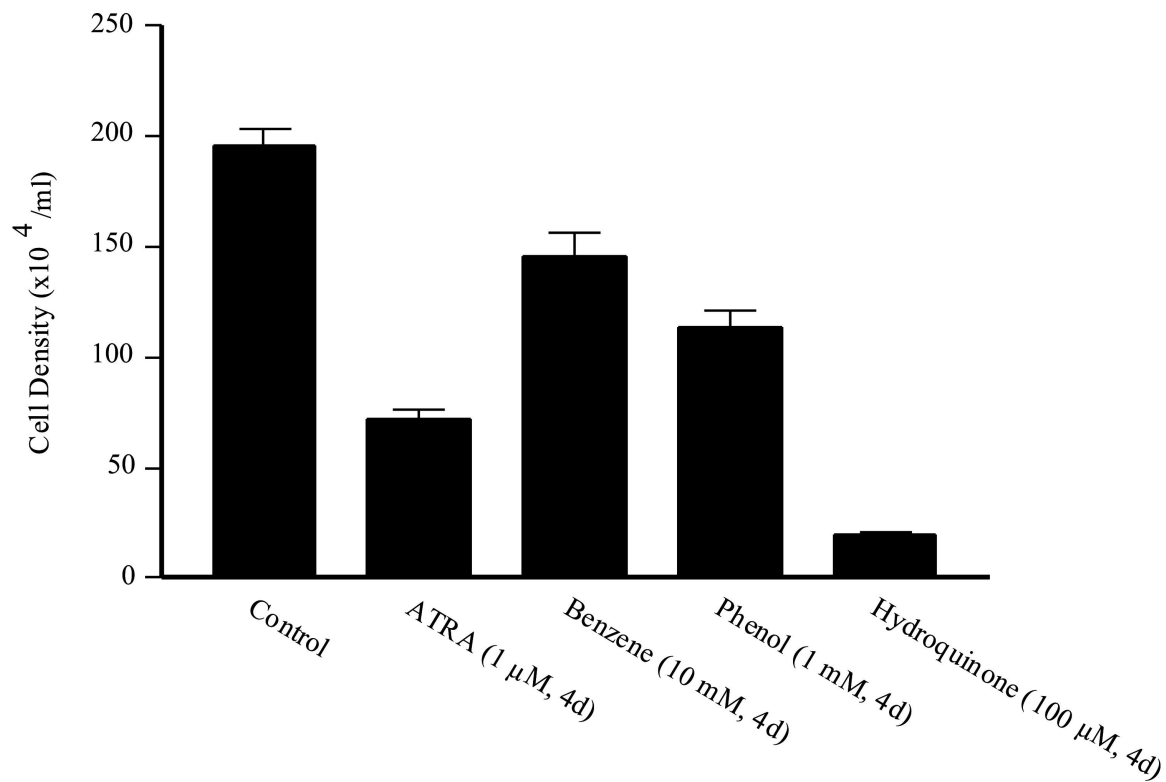
Figure 2C

Figure 2D

Figure 3
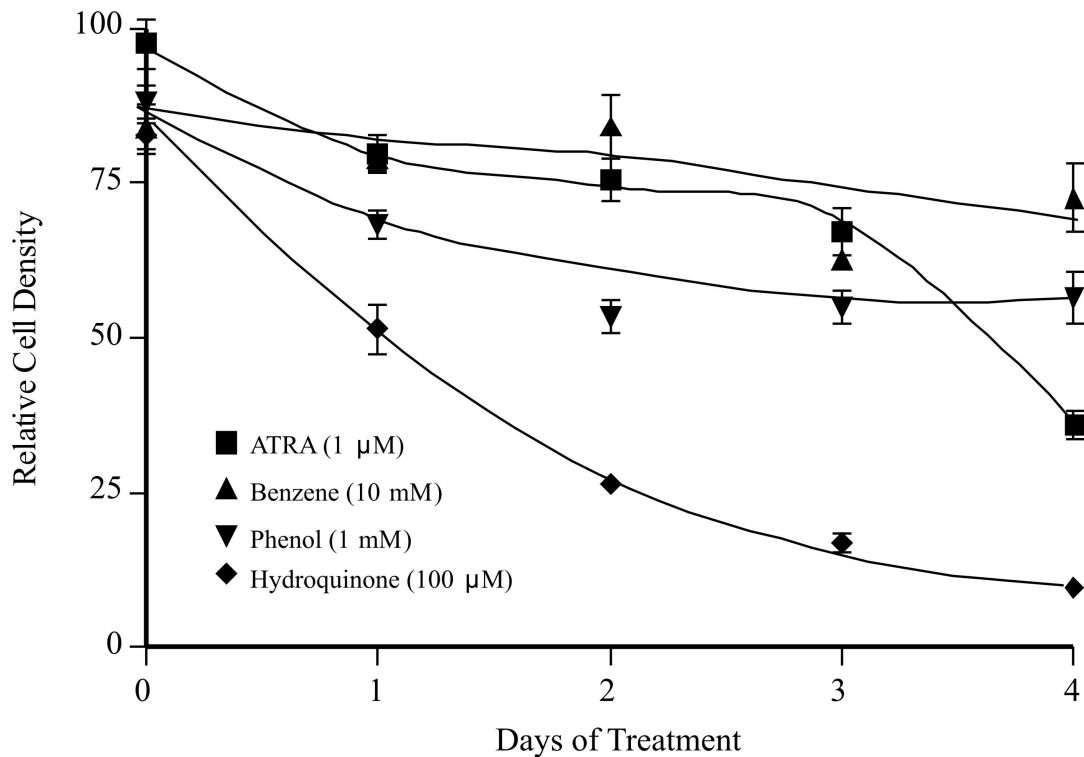
Figure 4
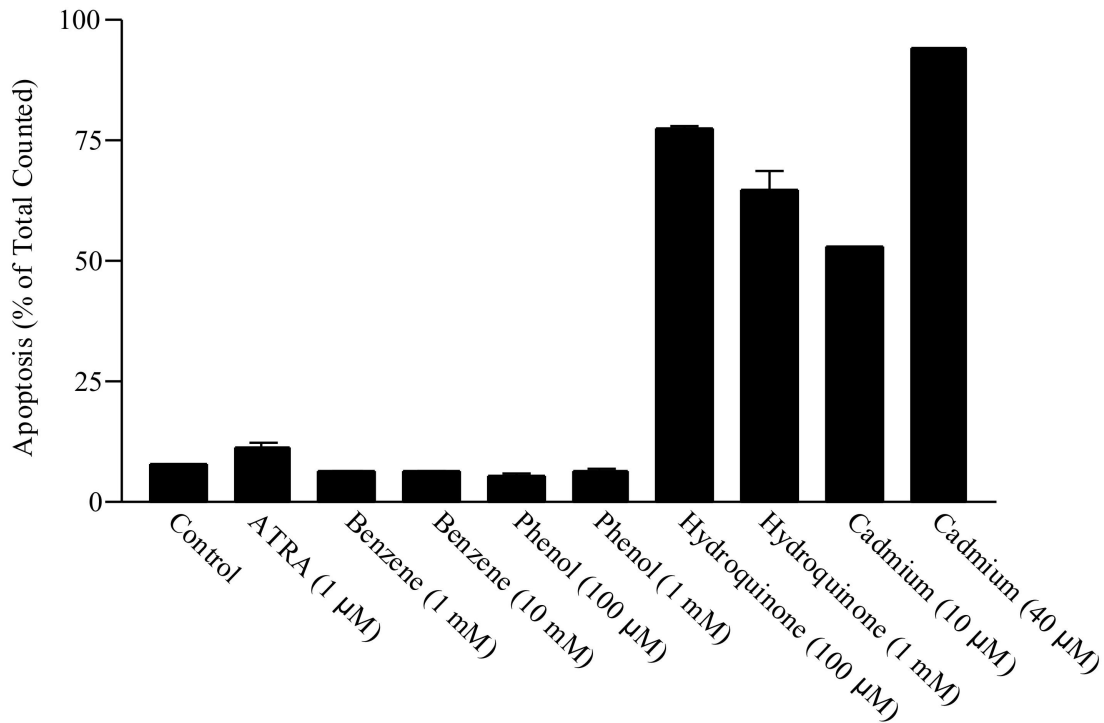
Figure 5

Control HL60 cells   ATRA 1 µM   Benzene 1mM   Benzene 10mM
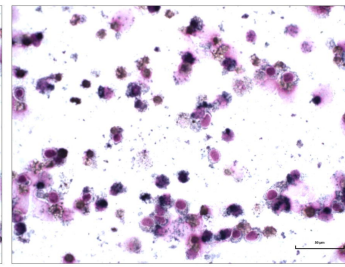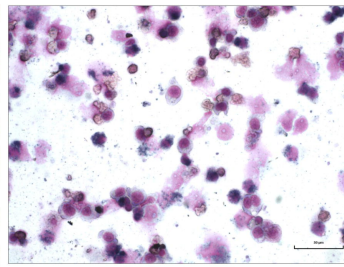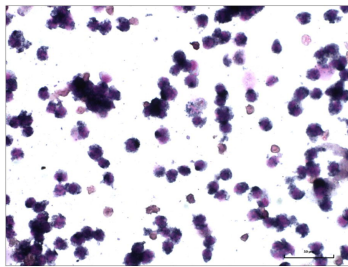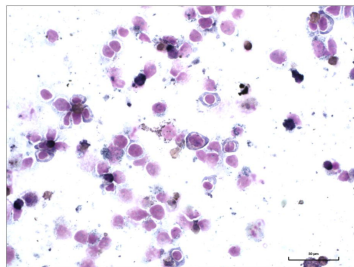
Phenol 100µM   Phenol 1mM   Hydroquinone 100 µM   Hydroquinone 1mM
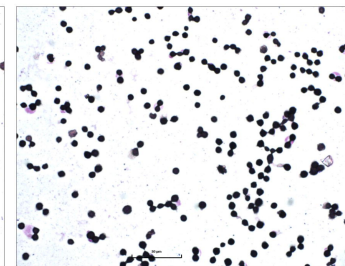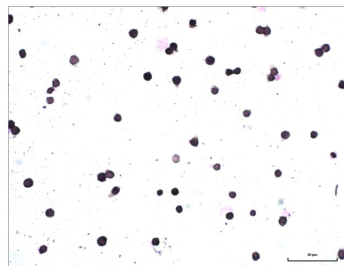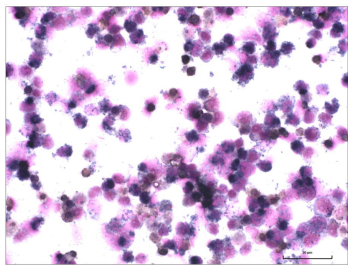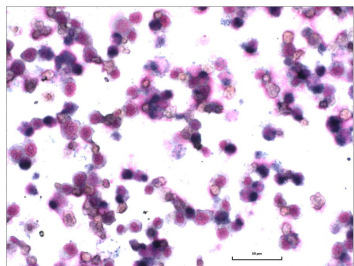
Figure 6A
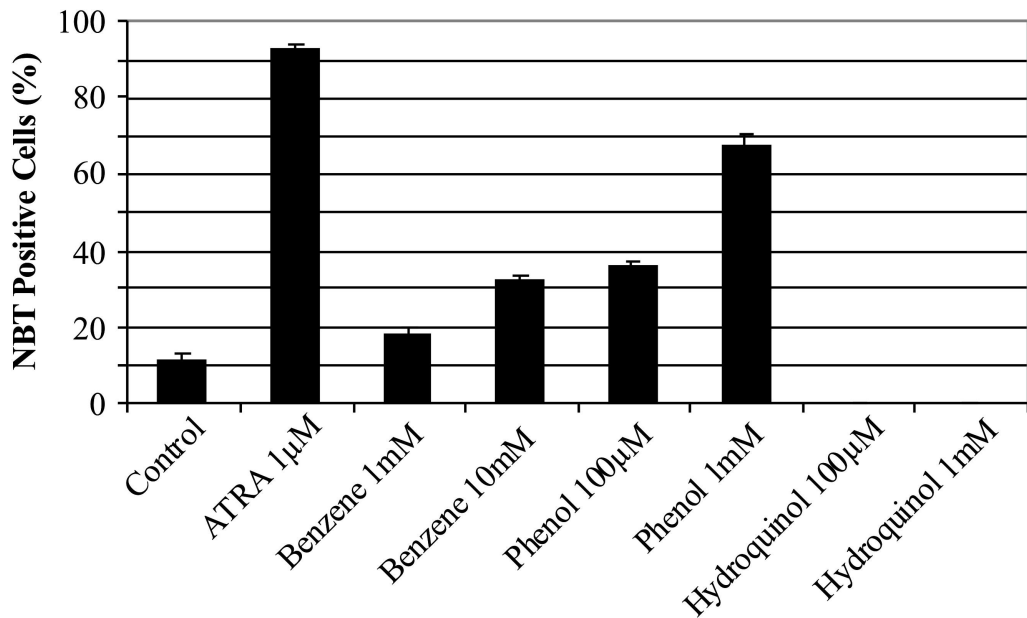
Figure 6B

Control

ATRA

Phenol

Figure 6C

Control   Phenol

55 kDa →   4F3

Actin
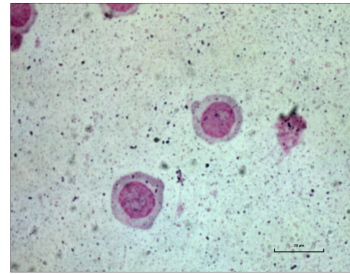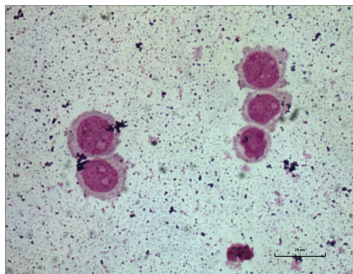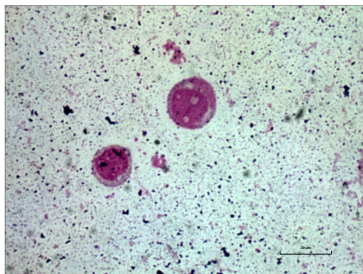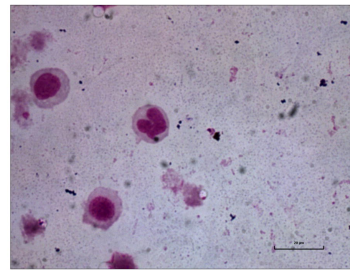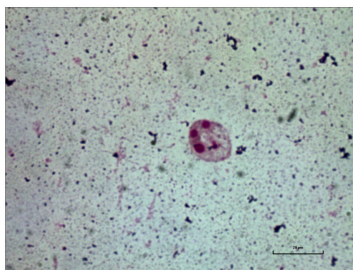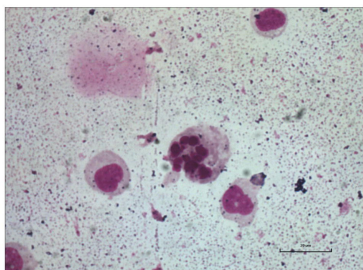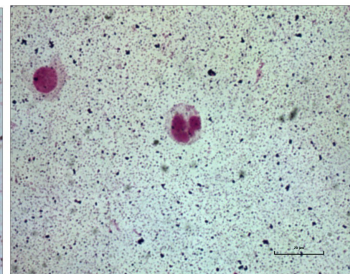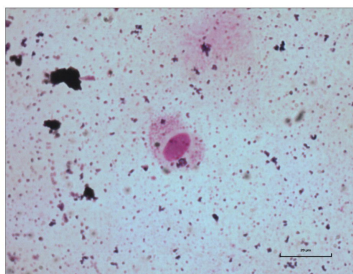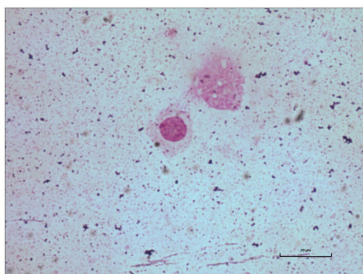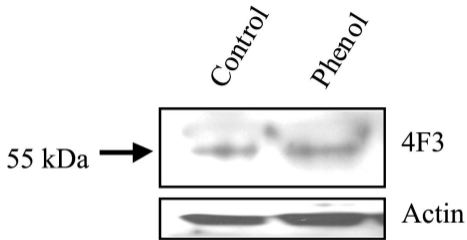
Figure 7A

Figure 7B

Figure 8A

55 kDa ➡️

Control Hemin Phenol

4F3

Actin
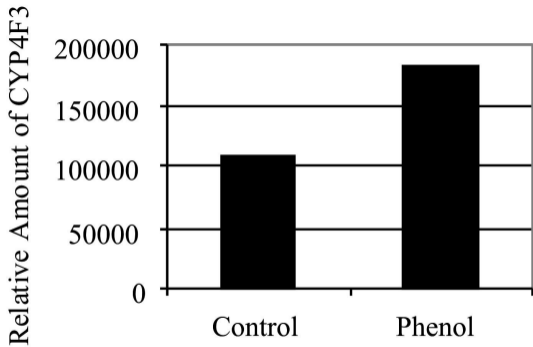
Figure 8B

Figure 8C

Figure 9A

Figure 9B