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Tubulin and other protein targets in HL-60 Cells exposed to ¹⁴C-hydroquinone

Darion Elizabeth Lawson
San Jose State University

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TUBULIN AND OTHER PROTEIN TARGETS IN HL-60 CELLS EXPOSED TO
¹⁴C-HYDROQUINONE

A Thesis

Presented to

The Faculty of the Department of Biological Sciences
San Jose State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

By

Darion Elizabeth Lawson

May, 1996

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APPROVED FOR THE DEPARTMENT OF BIOLOGY

Dr. Leon Yengoyan

Dr. Kenneth W. Turteltaub

Dr. Steven White

APPROVED FOR THE UNIVERSITY

Serena J. Stanford

ABSTRACT

TUBULIN AND OTHER PROTEIN TARGETS IN HL-60 CELLS EXPOSED TO ¹⁴C-HYDROQUINONE

By

Darion Elizabeth Lawson

Benzene, a constituent of cigarettes and gasoline, is a well known hematotoxin causing many blood disorders, including cytopenia, aplastic anemia and leukemia. The mechanism of benzene induced myelotoxicity and leukemia in humans is unknown. It has been suggested that toxicity may be caused by covalent binding of one or more metabolites to cellular macromolecules and that tubulin may be one of these cellular targets. The purpose of this study was to assess whether 1 μ M ¹⁴C-hydroquinone (HQ), binds to tubulin in HL-60 cells, using 1-D PAGE, 2-D PAGE, immunoblot and accelerator mass spectrometry (AMS). Results indicate that: 1) tubulin in these cells is a target for covalent modification under these conditions and, 2) there are a number of other protein targets. In addition, total protein binding in perchloric acid (PCA) precipitable protein may be non-linear at low exposure levels.

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INTRODUCTION

Production and Uses

Benzene is a common industrial chemical and a constituent of many commercial products. Production of benzene ranges between 11-12 billion pounds per year (1). Benzene was first isolated in 1825 by the compression of oil gas. Early production of benzene first occurred in 1849 when it was obtained from light oil derived from coal tar; and later in 1941 when it was first extracted from petroleum (3). After that time, it was used quite extensively as a solvent for the production of rubber and adhesives. Some of the more common uses of benzene include the synthesis of polystyrene, as a solvent for ink, and as an anti-knock compound for gasoline. Currently, the most common use of benzene is in the manufacture of paints and plastics.

Occupational and Environmental Exposure

Benzene has long been recognized as a chemical hazard. Shortly after it was introduced as a solvent for adhesives, effects related to toxicity were observed and recorded. In 1913, Alice Hamilton began a campaign to increase safety measures relating to benzene use. In 1918, xylene began to be used as a replacement for benzene. It wasn't until the mid 1940's, though, that occupational exposure limits began decreasing.

In 1987, the Occupational Safety and Health Administration (OSHA) lowered the time weighted average (TWA, Appendix 4) for benzene exposure to 1 ppm from a previous level of 10 ppm. The short term exposure limit (STEL, Appendix 4) was also lowered to 5 ppm (Appendix 4). Due to regulatory

stringency focused on benzene exposure in the workplace and also because benzene is a constituent of both gasoline and cigarettes, the "at risk" population has shifted to a much larger constituency who experience a lower level of exposure. In the United States, gasoline contains an average of 0.8% benzene and in Europe an average of 5% (1, 2). Studies indicate that the smoke from a single cigarette may contain 47-64 ppm of benzene (3). Subsequently, smoking may be identified as a significant source of benzene exposure to the 50 million people in the United States, alone, who smoke. Because approximately one-half of the benzene inhaled is excreted by exhalation (1), included in this "at risk" group are those who are exposed to second-hand smoke.

Toxicity

Most of the literature addressing the toxic effects of benzene in humans take the form of epidemiological studies and case reports. Typically, symptoms associated with acute benzene exposure are central nervous system related. These include drowsiness, dizziness, headache, delirium and loss of consciousness; and are correlated with occupational exposure levels of between 30 ppm to 300 ppm (1). Exposure to concentrations of 19,000-20,000 ppm for 5-10 minutes is fatal. Chronic exposure to elevated levels of benzene (above 50-100 ppm) is associated primarily with hematotoxic effects in humans and a number of different carcinomas in animals (1). In humans, decreased cellularity in the blood is the first indication of toxicity. Continued exposure often results in the development of pancytopenia (a decrease in all cell types), then aplastic anemia (inability of stem cells to differentiate), and in many cases progresses to

leukemia. If the toxic effects have not progressed past aplastic anemia, they are often reversible when exposure is ceased.

Blood disorders relating to benzene exposure were recognized as early as 1897 when nine patients were studied at the University of Stockholm. These patients exhibited chronic benzene toxicity and regularly used benzene occupationally. Four of the patients died from aplastic anemia (1). One of the well known epidemiological studies linking benzene exposure to leukemia occurred in 1974. In this study, Muzaffer Askoy described an increased incidence of leukemia and preleukemia in shoe slipper and handbag workers. Rates of leukemia and preleukemia in this population were more than twice that observed in the general population (reviewed in 1 & 2). At the time the study was performed, benzene levels in the air at the factories were as high as 210 ppm to 650 ppm. There are many additional epidemiological and case reports which indicate a similar connection (1, 2).

Animal studies present a different picture of benzene toxicity. Although there is a multitude of animal studies, only some of the effects observed in humans have been reproduced in animals. Some of the many types of cancers induced in animals by chronic exposure to benzene include zymbal gland carcinoma, lymphoma, mammary gland carcinoma, hepatoma, and nasal carcinoma. None of these effects has been observed in humans. Cronkite exposed CBA/Ca mice to 300 ppm benzene for 16 weeks in a manner which mimicked occupational exposure and found a significant increase in the number of mice with leukemia from the exposed group versus the control group (reviewed in 1 & 2). Another study, done a few years previous by Goldstein in 1982, where

CD-1 mice were exposed to 300 ppm benzene for life, reported only a possible correlation between exposure to benzene and the incidence of leukemia.

Although no strong correlation between benzene exposure and leukemia has been made in animals, injury directed towards the hematopoietic organs, similar to that occurring in humans, is a common effect observed in many species. These effects include leukopenia, lymphocytopenia, granulocytopenia, and pluripotent stem cell depletion (all reviewed in 1 & 2). Subsequently, there is not a good animal model for benzene induced human leukemia.

Distribution

Benzene is rapidly absorbed into the body after inhalation exposure (1, 2). After exposure of humans to 52 to 62 ppm, 50% of an inhaled benzene dose is exhaled unchanged, while 30% is retained in the body (1, 2). The remainder is excreted in the urine or feces either unchanged or as metabolic products (1, 2).

Because it is a highly lipid soluble and neutral molecule, benzene primarily deposits in tissues with high levels of fat. Tissues which have been shown to contain the highest levels of benzene after exposure include fat, bone marrow, kidney and liver. Fifty percent of the retained dose is found primarily in bone marrow, fat and liver (1, 2).

The perfusion rate of an organ is also a major factor in distribution of benzene. Highly perfused organs such as the kidney, liver and brain tend to have higher concentrations of benzene after inhalation exposure. The target for toxicity is the hematopoietic tissue, namely the bone marrow. It is believed that metabolites accumulate in this organ (1).

Metabolism

Metabolism of benzene is required for toxicity. Partial hepatectomy in rats protected against depressed erythropoiesis (1, 2). Although bone marrow is the target organ for toxicity in humans, metabolism of benzene occurs primarily in the liver and to a much lesser degree in the bone marrow. The first step in the metabolism of benzene is the conversion to phenol via an unstable benzene epoxide or benzene oxide oxepin intermediate. Hydroxylation to phenol in liver microsomes is catalyzed by the cytochrome P450IIE1 isozyme (8). Further oxidation of phenol, by cytochrome P450IIE1 in the liver, yields hydroquinone and catechol. Metabolism of these two products yields *p*-benzoquinone, *o*-benzoquinone and trihydroxybenzene (6). In addition, the formation of muconaldehyde and muconic acid occurs via either an intermediate benzene oxide or benzene oxide oxepin

In the bone marrow, however, metabolism of phenol to hydroquinone and catechol is believed to be catalyzed by a myeloperoxidase enzyme (24, 40). Subsequent metabolism, by unknown mechanisms, of both of these compounds yields benzenetriol, *p*-benzoquinone and other quinones and semiquinones. It has been observed that the bone marrow is able to accumulate these metabolic products. Peak metabolite levels in the bone marrow have been observed to be nine times that in blood (1, 2). In addition to those compounds mentioned, urinary products of benzene metabolism include metabolite conjugates of sulfates, esters, and glucuronides (1, 2).

Studies indicate that certain metabolic products produced oxidative damage to cellular macromolecules. It is believed that this damage is a result of one or more reactive intermediates formed during metabolism.

Figure 1. Metabolic Pathways for Benzene*

* Taken from review by Snyder, Witz, and Goldstein, 1993.

Mutagenicity

Benzene has not been shown to be mutagenic using standard microbial mutagenicity assays, *Drosophila melanogaster* in a sex linked genetically unstable test system, or in the mouse lymphoma forward mutation assay. The mutagenic capability of benzene metabolites, though, has been documented. Catechol, hydroquinone and 1, 2, 4 trihydroxybenzene have all been found to be mutagenic in V79 cells (40).

Benzene has been shown to be clastogenic in mammals *in vivo* and *in vitro* (1-3,16,17). Damage observed include an increased incidence of chromosomal aberrations including sister chromatid exchange (SCE), polyploidy and chromatid breaks (reviewed in 2). In addition, micronuclei formation in erythrocytes after exposure to benzene and its metabolites *in vivo* (37, 38); and in lymphocytes after *in vitro* exposure to one or more metabolites (16, 17, 18) has been reported. Two substances which compete for free radical binding, ascorbic acid and glutathione, both protect against DNA damage (reviewed in 2).

Covalent Binding

Benzene and its metabolites are capable of binding covalently to a number of macromolecules both *in vitro* and *in vivo*. Macromolecular adduct formation is believed to occur via a free radical formed during metabolism (1-3, 40). Covalent binding via free radical formation occurs with other chemical toxicities such as that observed with carbon tetrachloride (41). For example, hydroquinone and *p*-benzoquinone inhibit mitochondrial DNA replication *in vitro* (6). This effect has been attributed to *p*-benzoquinone reacting with sulfhydryl groups on DNA

polymerase gamma. Tunek, in 1979, described a 55 kDa protein target for binding after exposure to benzene in liver microsomes using 1-D PAGE and fluorography (11). Studies by Irons and colleagues (20, 21, 22) have found that a number of benzene metabolites inhibited polymerization of the alpha and beta subunits of tubulin in *in vitro* cell free assays. A recent study by Soucek suggested cytochrome P450 to be another target (42). Although it is probably not the only feature, it is possible that protein binding may be a feature in the development of hematotoxic effects and leukemia. It must be stated, although, that this mechanism is as yet unproved.

Accelerator Mass Spectrometry

Accelerator mass spectrometry (AMS) is a technology used to measure isotope ratios (35, 36). It was originally developed approximately 15 years ago for use in archaeology and geochronology studies for counting radiocarbon.

Carbon in the form of graphite is ionized using a cesium ion sputtering source which produces carbon ions in a (-)1 charged state. The selected ions are then accelerated by a potential of 6.5 MeV and passed through a foil to strip the outer shell electrons. Using magnets to direct the carbon path, the beam is "steered" along two turns. Because different isotopes of the same element have different masses, ^{13}C and ^{12}C are separated from the beam. ^{13}C is collected in a Faraday cup and serves as the denominator of the ^{14}C to ^{13}C ratio. Finally, the isolated ^{14}C is detected by a selection based on momentum, energy velocity and energy loss.

Recently, this technology has been adapted for analysis of biomedical samples. The sensitivity limit is on the order of attomole of ^{14}C /milligram of

carbon. 1.0 Modern is equivalent to 98 amole ^{14}C per milligram carbon. For example, 0.1 Modern is approximately 1% of natural abundance levels of ^{14}C in the human body. The unit Modern has been used in radiocarbon dating for many years. It is defined to be 0.95 times the radiocarbon activity in an oxalic acid standard from the U.S. NIST (36). It is an approximation of the amount of ^{14}C in the atmosphere in 1950. With sensitivities as low as this, it is possible to measure radioisotopes just slightly above natural abundance levels. For this study, AMS will allow the characterization of binding patterns in proteins exposed to low levels of ^{14}C -HQ.

Experimental Rationale

In this study, I investigated whether hydroquinone, a metabolite of benzene, covalently binds to tubulin in HL-60 cells when exposed to environmentally relevant levels. Hydroquinone is a secondary metabolite of benzene and is one of the metabolites believed to play a role in the development of benzene-induced leukemia. HL-60 cells were originally isolated from a person with acute myelocytic leukemia. They are believed to have been derived from the promyelocytic stage in blood cell development. Since it is believed that benzene-induced leukemia development occurs during one of the early stages in cell differentiation, these cells will provide a good model for investigating effects of hydroquinone exposure as it relates to protein adduction. Covalent binding of HQ to tubulin has not been studied after exposure to the cell system itself. Of the studies known, characterizing tubulin as a target for covalent modification by benzene or its metabolites, all have used tubulin subunits in cell free assays.

After exposure, separating proteins by 1-D and 2-D PAGE (Appendix 4) essentially isolates the ^{14}C activity to that which was bound to protein. Free ^{14}C -HQ is electrophoretically removed or diffuses out of the gel. Separating proteins by 1-D PAGE and cutting each lane into segments allows for the determination of the molecular weight ranges which exhibit increased levels of ^{14}C activity. Samples can also be separated by 2-D PAGE which separate by isoelectric point in the first dimension and molecular weight in the second dimension. By using immunoblotting to locate the alpha and beta subunits of tubulin, the spots can be removed and measured for ^{14}C activity. Thus, whether tubulin is truly being targeted for covalent modification in a cellular system can be ascertained.

Having a better understanding of the mechanism by which benzene acts to cause leukemia and other hematotoxic effects, it may be possible to gain insight into the induction of carcinogenesis and development of toxicity, and how to counteract or prevent the onset of benzene-induced leukemia.

FIGURE 2. Schematic of the LLNL Accelerator Mass Spectrometer*

Schematic of the LLNL accelerator mass spectrometer shows the major components used to obtain part in 10^{15} sensitivity in detecting rare radioisotopes. This spectrometer, based on a rebuilt Van de Graaff accelerator, is large (accelerator length ca. 13.5 m), but smaller systems also exist. The accelerator provides energy gain and dissociates isobaric molecular interferences by stripping two or more electrons from negative ions as they pass through a thin carbon foil or diffuse gas in the center of the accelerator.

*Taken from Vogel and Turteltaub, 1992

MATERIALS AND METHODS

Cell Culture Conditions

HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in 25 cm² flasks. Cells were grown using 10 mls of culture media containing RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO), 1% L-glutamine (Gibco, Gaithersburg, MD), 100 units/ml penicillin (Gibco, Gaithersburg, MD) and 100 µgrams/ml streptomycin (Gibco, Gaithersburg, MD). Culture conditions were maintained at 37°C in a 95%/5% air/CO₂ humidified atmosphere.

Cell Viability

Cell viability was determined using the Trypan Blue dye exclusion method, using 0.4% Trypan Blue dye solution (Gibco, Gaithersburg, MD) prior to harvesting. Cells were pooled to yield 4-5 samples and between 100-300 cells were counted per sample. The number of cells used in each experiment is stated as the number of viable cells.

¹⁴C-Hydroquinone Radiopurification

Radiopurity of the ¹⁴C-hydroquinone (Wizard Laboratories-Davis, Ca) solution was determined by reverse phase chromatography using a Rainin HPXL high pressure liquid chromatography (HPLC) system with a Dynamax UV

detector. For analysis, a Dynamax analytical column with a 0.46 mm ID and 25 cm length was used. Absorbance was measured at 270 nm.

When analyzing samples, 5 μ l of the ^{14}C -hydroquinone (^{14}C -HQ) solution was run isocratically at 100% 20 mM phosphate buffer, pH 4.0 (Appendix 1). Fractions were collected at 1 minute intervals and radioactivity was measured using a Wallach 1410 liquid scintillation counter to correlate the UV detected peaks with radioactivity in each fraction.

Radiopurification was accomplished by using the same Rainin HPL system under the same conditions as for analysis, but a Dynamax semi-preparative column with a 10mm ID and 25 cm length was used in place of the analytical column. Five fractions were collected on ice and under nitrogen to prevent oxidation. The samples collected were as follows: #1 was from 0-15 minutes, #2 was just prior to where the ^{14}C -HQ eluted (16-22 mins) #3 was the ^{14}C -HQ peak (23-30 mins), #4 included material just after the ^{14}C -HQ peak and the wash (30-35 mins of the run and 1-22 mins of the wash), and #5 was the tail end of the wash (22-28 mins). The final purified ^{14}C -HQ solution (sample #3) was stored immediately at -20°C under nitrogen.

The concentration of the purified ^{14}C -HQ was determined by spectrophotometric analysis using known concentrations of unlabeled hydroquinone as the standard (Appendix 2). Absorbance was measured at 270 nm. Final concentration of the solution was determined to be 0.7543 mM. The extinction coefficient was calculated to be 267 % $\cdot$ L/mol $\cdot$ cm. The sample was stored at -20°C under nitrogen when not in use.

Total Protein Binding Analysis

Eighteen samples, each containing 7×10^6 viable cells, were harvested and washed once in 37°C RPMI 1640, then resuspended in 10 ml of RPMI 1640. Samples were equilibrated for 10 mins at 37°C in a 95%/5% air/CO₂ humidified incubator. ¹⁴C-hydroquinone was added to final concentrations of: 0 nM, 0.37 nM, 0.76 nM, 11.2 nM, 108.9 nM, and 1.05 μM. 20 mM phosphate buffer, pH 4.0 was added to make the total volume added equivalent. Cells were exposed for one hour at 37°C in a 95%/5% air/CO₂ humidified incubator. Each exposure concentration was run in triplicate.

After exposure, each sample was washed four times in phosphate buffered saline (PBS), pH 7.4, the final cell pellet was resuspended in 1 ml of 7% PCA (Appendix 2) (to precipitate out macromolecules from solution), and centrifuged at 2500 rpm in an IEC tabletop centrifuge. The pellet was then washed a second time in 5% PCA. After a second centrifugation, the pellet was washed twice in 50% methanol (to remove salts, DNA, and RNA), then washed twice in a 1:1 ratio of ethanol:diethyl ether (to remove lipids). The protein pellet was harvested by centrifugation after each wash, as described previously. The final protein sample was resuspended in 0.5 mls of 0.1 M potassium hydroxide (to resolubilize protein) and incubated overnight at 37°C.

50μl of each sample was scintillation counted to determine the ¹⁴C activity. The samples with low enough activity to be non-detectable by scintillation counting were analyzed by AMS. After determining the protein concentration in each AMS sample using a Bradford protein assay (Appendix 2) (Pierce), 2 mg of tributeryn was added to each. They were dried in a Savant vacuum centrifuge

and prepared for analysis. Samples exposed to 0 nM, 0.37 nM, and 0.76 nM ^{14}C -hydroquinone were analyzed by AMS.

1-D PAGE Analysis

Two samples, each containing 25×10^6 viable cells were washed once with RPMI 1640, after harvesting by centrifugation at 2500 rpm (500 x g) in an IEC tabletop centrifuge at room temperature for 12 minutes. Cells were resuspended in 10 ml of 37°C RPMI 1640 and equilibrated in a humidified incubator for 10 minutes at 37° C and 95%/5% air/CO₂. The viable cell concentration was 2.5×10^6 cells/ml. 13.2 µl of a 0.7543 mM ^{14}C hydroquinone solution was added (0.221 µCi). Final ^{14}C -hydroquinone concentrations were 0 µM and 1 µM. After one hour of exposure, cells were harvested by centrifugation, as noted previously, and washed two times in 10 mls of phosphate buffered saline(PBS), pH 7.4 (Appendix 1). 100µl of the dosed cell media and 100 µl of the supernatant from each of the washes were saved for scintillation counting. The cell pellet was resuspended in sodium dodecylsulfate (SDS) buffer (Appendix 1), then centrifuged in an Eppendorf microfuge at 14,000 rpm for 30 minutes.

Samples solubilized in SDS buffer were separated by one dimensional gel electrophoresis by the methods described in Current Protocols (43). Protein concentrations for SDS solubilized samples were not obtained. 20 µl of each sample was loaded into a well and electrophoresed overnight at 20 mA. When the run was complete, the gel was stained in a Coomassie blue dye solution (Appendix 1) for 1 hour. The gel was then destained in destain solution (Appendix 1) until the background disappeared. The image was captured using

image capture hardware connected to a PC loaded with RFLP® image analysis software.

Six additional lanes were run with four containing different amounts of bovine serum albumin (BSA) (Sigma, St. Louis, MO) standards (1 µg, 2 µg, 5 µg, and 10 µg), one containing molecular weight standards (14.2 kDa-200 kDa) (Bio-Rad, Hercules, CA), and one containing the same volume of undosed HL-60 protein. These extra lanes were used to determine the molecular weight and concentration of the protein in each segment analyzed.

The lanes containing undosed and dosed electrophoresed protein to be analyzed, were cut into 62 and 64 segments, respectively. To each segment was added 2 mg of tributeryn as carrier carbon to aid in the combustion of the sample. They were then dried in a Jouan vacuum centrifuge. Each segment was combusted, converted to graphite, then analyzed by accelerator mass spectrometry for ^{14}C activity.

2-D PAGE Analysis

Six samples of 30×10^6 cells were washed once with RPMI 1640 after harvesting cells by centrifugation at 2500 rpm (500 x g) in an IEC tabletop centrifuge at room temperature for 12 minutes. Cells were resuspended in 10 ml of 37°C RPMI 1640 and equilibrated in a humidified incubator for 10 minutes at 37° C and 95%/5% air/CO₂. The viable cell concentration was 3×10^6 cells/ ml. 13.2 µl of a 0.7543 mM ^{14}C hydroquinone solution was added (0.221 µCi). Final ^{14}C -hydroquinone concentrations were 0 and 1 µM for each exposure group and was run in triplicate. After a one hour exposure, cells were harvested by centrifugation, as noted previously, and washed four times in 10 mls of

phosphate buffered saline (PBS), pH 7.4. 100µl of the dosed cell media and 100 µl of the supernatant from each of the washes was saved for ^{14}C activity determination by scintillation counting. After washing the cells, the pellet was resuspended in 100 µl Urea/NP-40 buffer (Appendix 1). The samples were passed 10 times through a 22 ga needle attached to a 1cc syringe to shear the DNA. Next, the solubilized protein sample was ultracentrifuged (200,000 x g) for 50 minutes at 24°C to sediment DNA.

Proteins were separated by two dimensional gel electrophoresis by the method of Anderson, et al. (28). Separation by isoelectric point (pI) was performed in the first dimension and molecular weight in the second dimension. 40 µl of sample was loaded onto 1.5 mm diameter 5.3% acrylamide tubes for separation by pI using a pH range of 4-8. Proteins were electrophoresed overnight at 14,000 volt-hours. The second dimension was run on a 15% acrylamide gel at a constant 600 mA with molecular weight markers added to a well created in the gel on the acidic end.

Immunoblotting

When electrophoresis was complete, gels were equilibrated for 15-30 minutes in Towbin transfer buffer (Appendix 1). Proteins were transferred, by the method of BioRad (34), onto a 0.45µ pore size nitrocellulose membrane for 50 minutes at 50 volts. When transfer was complete, nitrocellulose membranes were blocked with Tween-20/tris buffered saline (TTBS) (Appendix 1) for 1-3 hours. Membranes were incubated in 30 mls of an antibody solution containing a dilution of 1:1000 anti-alpha tubulin (Zymed) and 1:250 anti-beta tubulin (Zymed) in TTBS. Incubation continued overnight. Membranes were washed 5 times in

PBS then incubated for 1-3 hours in rat anti-mouse IgG secondary antibody with horseradish peroxidase conjugate (Zymed) at a dilution of 1:250 in TTBS (Appendix 1). Membranes were then washed 5 times in PBS and incubated with SigmaFast diaminobenzidine (DAB) solution (Sigma, St. Louis, MO). The reaction was allowed to continued until it was observed that the color had developed completely. The membranes were, then, rinsed 5 times in water.

Each spot on the membrane was cut out using a scalpel and to which each was added 2 mg of tributeryn as carrier carbon to aid in sample combustion while making graphite. Samples were dried in a Jouan vacuum centrifuge, combusted, and converted to graphite as describe in the next section; then analyzed by AMS for ^{14}C activity, as described previously.

Accelerator Mass Spectrometry (AMS)

The assumption in measuring these samples by AMS is that the levels of ^{14}C measured in the dosed samples, which is above those observed in the undosed samples, is due to ^{14}C bound directly to protein. During electrophoresis, it is assumed that unbound ^{14}C labeled compound will either diffuse out of the gel into the electrophoresis tank solution or be electrophoretically pulled along with the dye front. For experiments determining total protein binding, it is assumed that the multiple washes in acid, methanol, and ethanol:diethylether effectively remove all residual unbound ^{14}C labeled compound and that the ^{14}C measured is only that which is bound to protein.

Samples were combusted at 750°C for 2 hours to form CO_2 . Reduction of CO_2 to graphite occurred by combustion, first, at 500°C for 3 hours and, then, at

550°C for 2 hours. This was the final sample form which underwent analysis by AMS.

Data Presentation

Total Protein Binding

Results are presented as μ moles of ^{14}C -hydroquinone per milligram of protein. For the samples analyzed by AMS, protein concentrations were determined by method of Bradford. Protein concentrations were not determined for samples counted by scintillation counting. Because both AMS and scintillation counted samples were treated and isolated in concert, the average protein concentration of the AMS samples was used as the putative protein concentration for these calculations. The amount of carbon in mammalian protein was previously determined in this laboratory by another researcher.

1-D PAGE

The amount of protein in each segment from the 1-D gels was determined using RFLP[®] image analysis software. Varying amounts of bovine serum albumin was used as the concentration standard. The amount of ^{14}C activity in each segment is presented as fmole of $^{14}\text{C}/\mu\text{gram}$ of protein.

2-D PAGE

In each of the dose groups, exposed and unexposed, the results are presented as average fraction Modern among antibody bound spots in 46-69 kDa

range versus average fraction Modern among non-antibody bound spots. It was not possible to quantitate the amount of protein in each spot obtained.

RESULTS

¹⁴C-Hydroquinone Purification

Data from HPLC characterization of ¹⁴C -hydroquinone is presented (Fig. 3). As observed, ¹⁴C activity in the collected sample correlates with the peak observed at approximately 8 minutes when measured at 270 nm. A₂₇₀ was determined as the optimum wavelength by obtaining a 200-700 nm UV-VIS spectrum using an unlabeled 1 mM hydroquinone solution. A 200-700 nm UV-VIS spectrum of the purified ¹⁴C-hydroquinone was compared to the cold HQ solution to affirm the purity. They were observed to be identical.

Total Protein Binding:

The amount of ¹⁴C-HQ binding, to PCA precipitable protein isolated from HL-60 cells exposed to various concentrations of ¹⁴C-hydroquinone, was determined by AMS and scintillation counting. Radioactivity in samples was initially measured by scintillation counting and those with activity below 1 dpm, after subtracting out background activity levels from reagents, were measured by AMS.

There appears to be a dose dependent increase in protein binding (Table 1) (Fig. 4a/4b). This data indicates that binding of ¹⁴C-hydroquinone to protein in HL-60 cells, at the exposure concentrations investigated, appears to exhibit a multiphasic pattern. At the low dose level (<1 nM), binding is non-linear (Fig. 4a), indicating a nearly logarithmic increase over the exposure levels presented. A fourteen-fold increase in ¹⁴C-hydroquinone dose produced a 875-fold increase

in binding. Finally, at exposures above 10.0 nM, this relationship appears linear ($r^2=0.98$) (Fig. 4b).

Figure 3
HPLC Trace of Radiopurified ^{14}C -Hydroquinone Correlating ^{14}C Activity to Peaks in Absorbance

Table 1

Total Protein Binding

¹⁴C-Hydroquinone Dose (nM)	Avg. nmoles HQ bound/mg Protein (±std. error) (x10⁻³)
0	0.006051±0.00148*
0.37	0.05044±0.03554*
0.76	0.1256±0.01181*
11.2	43.931±75.382**
108.9	3250.42±204.19**
1050	20412.53±2053.16**

n=3

*counted by AMS

**counted by scintillation counting

Figure 4a

14-C Hydroquinone Dose vs. Total Protein Binding

Figure 4b

1-D PAGE

After exposing cells to 1 μ M ^{14}C -hydroquinone, and separating protein by 1-D PAGE, several regions of increased ^{14}C activity are observed (Fig. 5) after standardizing the data for the amount of protein in each segment. Increases in ^{14}C activity are observed at molecular weights of 192 kDa, 158 kDa, 90-100 kDa, 75 kDa, 65 kDa, 58 kDa, 48-50 kDa, 37-40 kDa 25-32 kDa, and 13-19 kDa.

In the lower molecular weight range, the molecular weights of the specific targets are less easily determined because: 1) the amount of ^{14}C activity in some segments are as high as the background levels in segments from the high molecular weight range (samples are from the same lane) and 2) there are no distinct "peaks," but rather grouped segments which contain higher ^{14}C activity than that of the background. In the low molecular weight range, the background levels are as much as 50-70% lower than the observed "peaks." Possible low molecular weight targets are observed at molecular weight ranges of 37-42 kDa, 24-31 kDa, and 14-19 kDa. The increases in ^{14}C activity in segments from the exposed proteins are as much as 500-fold larger than that observed in the corresponding segments from unexposed proteins (Fig. 6). These results indicate that there may be a number of protein targets for covalent modification in these cells when exposed to 1 μ M ^{14}C -hydroquinone.

Figure 5

^{14}C Activity in Exposed Proteins Separated by 1-D PAGE

Figure 6

^{14}C Activity in Unexposed Proteins Separated by 1-D PAGE

2-D PAGE

HL-60 proteins isolated from cells, both unexposed and exposed to 1 μ M 14 C-hydroquinone, were separated by two dimensional gel electrophoresis, transferred to nitrocellulose, and probed with monoclonal antibodies for the presence and location of the alpha and beta tubulin subunits. Developed spots, resulting from reacting diaminobenzidine with horseradish peroxidase conjugated to a secondary antibody, were cut out and analyzed for 14 C activity using AMS. There was antibody development observed in the region of 46-69 kDa and pI of 5.5-6.5. In addition, there was a small amount of antibody development outside of that region (Fig. 7). The average amount of 14 C activity, of antibody bound proteins in the 46-69 kDa range in each gel, was determined. The three data points from each exposure group were averaged and is presented as the average amount of activity for that dose (Table 2).

This data indicates that there is a 180-fold increase in 14 C activity in protein from exposed samples, which is recognizable by anti-alpha and anti-beta tubulin antibodies. Antibody development occurred primarily in the range of 46-69 kDa and pI of 5.5-6.5. The alpha and beta subunits of tubulin are very similar in size at approximately 55 kDa. It is reported that both subunits are very similar in pI also, migrating within the range of 5.2-5.7 (39). There was some antibody binding outside of this molecular weight and pI range, but it was not extensive. Antibody development in regions outside of the 46-69 kDa range (i.e. the location and presence of spots) was inconsistent from gel to gel.

Table 2
¹⁴C Activity in Antibody Bound Spots in 46-69 kDa Range

Segment**	¹⁴C-Hydroquinone Dose (μM)	Avg. Fraction Modern
Antibody bound	0	0.42 $\pm$ 0.014
Non-antibody bound	0	0.40 $\pm$ 0.010
Antibody bound	1.17	75.8 $\pm$ 6.34
Non-antibody bound	1.17	9.8* $\pm$ 6.78

*n=3; There was one sample which gave a skewed value of 84. Without this value the mean is 3.1 $\pm$ 0.41.

**non-antibody bound spots were taken randomly both within and outside of the 46-69 kDa range.

Figure 7

**Antibody Binding Patterns on Nitrocellulose after 2-D PAGE and
Immunoblotting of HL-60 Proteins**

Binding patterns of monoclonal anti-alpha and anti-beta tubulin antibodies on nitrocellulose membrane containing cellular protein from HL-60 cells. Note: the shadow along the left side of the image is an artifact created when scanning the membrane image.

DISCUSSION

Total Protein Binding

Data from these experiments indicate that protein binding is multiphasic. The data from the two lowest exposure concentrations appears to indicate a non-linear trend. At lower levels of exposure, ≤ 1 nM, it is possible that there may be a logarithmic increase in binding. A 14-fold increase in dose, from 0.76 nM to 11.2 nM, produces a 875-fold increase in binding. At exposure concentrations which are greater than 11.2 nMolar, binding appears linear.

There are a number of possible hypotheses to explain this behavior, including the presence of multiple binding sites, induction of enzymes by hydroquinone, or a synergistic effect between hydroquinone and one or more metabolic or oxidative products. Hydroquinone can either auto-oxidize or be metabolically activated by a peroxidase enzyme to *p*-benzoquinone. HL-60 cells contain a significant amount of myeloperoxidase, an enzyme which is capable of catalyzing this reaction. It has been reported that *p*-benzoquinone is more effective than hydroquinone at producing oxidative damage, experimentally (1,2,3). This information provides a possibility that hydroquinone may not necessarily be acting alone, but in concert with one or more metabolites

The data presented here is not extensive enough to confirm or deny any of the mechanisms described. To gain a better understanding of the protein binding behavior of hydroquinone, further experiments are necessary. At this time, though, this data indicates a multiphasic pattern which is dependent on concentration levels of hydroquinone.

1-D PAGE

There are a number of increases in ^{14}C activity observed when exposed proteins are separated by 1-D PAGE. Among those is a quite prominent peak at approximately 58 kDa. The alpha and beta subunits of tubulin have been reported to have a molecular weights of 53 kDa and 56 kDa, respectively. The amount of activity in the exposed versus the unexposed protein, at that same molecular weight, is 5.131×10^4 and 0.0753×10^4 femtomole ^{14}C /microgram of protein. This is a 68-fold increase in ^{14}C activity. The measured ^{14}C activity in the two gel segments surrounding the 58 kDa sample, from the exposed protein sample, is 75% lower than that segment itself. It has been reported previously that tubulin may be a target for covalent modification by one or more metabolites of benzene. This data helps support the possibility that tubulin is indeed a target for binding by hydroquinone. There also seem to be a number of other protein targets for covalent modification after exposure of these cells to 1 μM hydroquinone.

In the gel containing exposed proteins, the relative background levels of ^{14}C activity, and also the "peaks," decrease as the molecular weight range decreases. This may be caused by some of the smaller sized proteins becoming trapped at the high molecular weight end of the gel because of the large amount of protein loaded into the lane. Achievement of 100% protein separation is quite difficult and often proteins become "clogged" when a large amount of protein is loaded. It is clear, though, that the ^{14}C activity observed at 192 kDa is not caused by trapped low molecular weight proteins. There were four gel segments ahead of that region which had a much lower level of ^{14}C activity. If the ^{14}C

activity in this ~192 kDa sample were due to diffusion or "clogging," a larger amount of ^{14}C activity would have been measured in those aforementioned samples. The amount of activity observed in the proteins from ^{14}C -HQ exposed cells is as much as 1000 times higher than observed in proteins from undosed cells.

Because an increase in activity is observed at approximately 58 kDa, this data suggests that one of the protein targets in these cells may be tubulin. Irons and Pfeifer in separate experiments first suggested that tubulin may be a target but did not investigate this effect after exposure to a cellular system. It also appears that there may be a number of other protein targets at this exposure concentration. For the purpose of this study, characterization of the other protein targets for binding was not completed. Continued analysis would be necessary to determine the identity of these proteins.

2-D PAGE

Using 2-D PAGE and immunoblotting with monoclonal antibodies specific to the alpha and beta subunits of tubulin, the location of these peptides was determined and the spots removed and analyzed for ^{14}C activity by AMS. The amount of ^{14}C activity in the anti-tubulin antibody bound spots in the unexposed proteins versus the 1 μM exposed was 0.4 Modern and 61.6 Modern, respectively. This represents a 154-fold increase in ^{14}C activity after exposure to 1 μM ^{14}C -hydroquinone. Samples were taken from other areas of the membrane to assess: 1) the background levels of ^{14}C in the membrane and 2) the possible presence of other protein targets. Background ^{14}C activity of nitrocellulose membranes containing proteins from unexposed cells, were quite low at

0.4±0.010 Modern. The background ^{14}C activity in membranes containing proteins from exposed cells had a mean of 9.8±6.78 Modern. This value is skewed because of one spot (out of twelve total) which had a level of 84 Modern. It is probable that this sample contains one of the other protein targets. If this is the case, this protein is approximately 20 kDa and has an approximate pI of 7-8. When determining background activity excluding this one data point, a value of 3.0±0.41 Modern is obtained.

Background ^{14}C activity differs between membranes containing exposed and unexposed proteins. The higher background in membranes containing exposed proteins is probably caused by exposure to residual unbound radiolabeled compound during the protein transfer process. Because this ^{14}C activity is so much lower than that observed in the antibody bound spots, it does not present a significant issue.

There was antibody development outside of the 46-69 kDa range. I believed that this is caused by a slight denaturation of the binding site on one or more of the antibodies when defrosting the solution prior to use. The solution was defrosted, quickly, in a 37°C water bath to increase the likelihood of the antibody recognizing the epitope. These other proteins may have similar epitope regions to those on the subunits of tubulin, allowing the antibodies to recognize the regions if they are slightly denaturation. To my knowledge, there is no information indicating that tubulin subunits have a molecular weight of other than 53-56 kDa. Many studies have separated and characterized multiple isoforms of both the alpha and beta subunits by PAGE. These studies indicate that although the isoelectric point of the isoforms might differ, the molecular weight range is essentially the same (39).

In conclusion, it appears that tubulin is indeed one target for covalent modification by hydroquinone in HL-60 cells exposed to 1 μ M concentrations. In addition, there are many other protein targets, one of which may be a protein of approximately 20 kDa with an approximate pI of 7-8.

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APPENDIX I

Reagents

[¹⁴C] Hydroquinone

[¹⁴C] Hydroquinone was purchased from Wizard Laboratories (Davis, California). Crystals were resuspended in a 20mM phosphate buffer solution, pH 4.0 to a final activity of 22.2×10^6 mCi/mmol.

20 mM Phosphate Buffer, pH 4.0:

2.28 mls of 85.6% phosphoric acid in a total volume of 1.0 liter
pH adjusted to 4.0 with concentrate potassium hydroxide

Urea/NP-40 Buffer:

9M Urea(BDH)
4% Nonidet P-40 (Sigma, St. Louis, MO)
2% Ampholytes 8-10 (Bio-Rad, Hercules, CA)
1% Dithiothreitol (Sigma, St. Louis, MO)

Towbin Transfer Buffer:

25 mM Tris Buffer (BDH)
192 mM Glycine (BDH)
20 % methanol (Baker)
pH should be about 8.1-8.3, but if it is off DO NOT adjust pH. Buffer must be remade

Phosphate Buffered Saline (PBS):

Make as a 10x solution and dilute to 1x as necessary by combining 100mls 10x to 900 mls water, final pH should be 7.4x
per liter, add:

80g NaCl (Baker)
2.0g KCl (Baker)
2.0g KH₂PO₄ (Baker)
21.6g Na₂HPO₄ (Baker)

TTBS

1% Tween 20 (Sigma, St. Louis, MO)
100 mM Tris(hydroxymethyl)methylamine (BDH)
0.9% sodium chloride (Baker)
2% bovine serum albumin (Sigma, St. Louis, MO)

SDS Buffer

2% sodium dodecylsulfate (BDH)
0.125 M Tris-Cl, (BDH)
2% dithiothreitol (Sigma, St. Louis, MO)
20% glycerol (BDH)

Coomasie Blue Stain Solution

50% methanol (Baker)

10% acetic acid

0.1% coomassie blue dye

Destain Solution

30% methanol

7% acetic acid

APPENDIX II

Assay Protocols

#1 Determination of Purified [14-C]hydroquinone Concentration Spectrophotometrically

Standard Curve:

20mM cold hydroquinone(HQ) was prepared using 20 mM phosphate buffer (PO₄), pH 4.1.

3.93 mg HQ was added to 1.79 ml phosphate buffer.

Calculations

3.93 mg HQ x mmole/110 mg x liter/20mmol = 1.79 ml phosphate buffer

<u>Concentration</u>	<u>Preparation</u>
20mM	see above
10mM	0.5ml of 20 mM HQ + 0.5 ml PO ₄ buffer
5 mM	0.5 ml of 10 mM HQ + 0.5 ml PO ₄ buffer
2.5mM	0.5 ml of 5 mM HQ + 0.5 ml PO ₄ buffer
1.25mM	0.5 ml of 2.5 mM HQ + 0.5 ml PO ₄ buffer
1.0mM	0.4 ml of 2.5 mM HQ + 0.6 ml PO ₄ buffer
0.5mM	0.5 ml of 1.0 mM HQ + 0.5 ml PO ₄ buffer
0.25mM	0.5 ml of 0.5 mM HQ + 0.5 ml PO ₄ buffer
0 mM	PO ₄ buffer

--each vial was wrapped in aluminum foil to prevent photooxidation

--spectrophotometer zeroed with 20mM PO₄ buffer, pH 4.0

--samples run in duplicate at 270 nm

--after standard concentrations measured machine zeroed again and with PO₄ buffer and ¹⁴C-HQ measured at 270 nm and compared against the standard curve.

#2. Protocol for Protein Isolation from HL-60 cells

Reagents:

5% perchloric acid(PCA): dilute a 70% stock solution to 1 in 14

50% methanol(MeOH): dilute in water

1:1 solution of diethyl ether and ethanol(Et₂O/EtOH)

0.1 M potassium hydroxide (KOH)

1 M , 0.1 M and 0.01 M hydrochloric acid (HCl)solutions

1. Suspend cell pellet in 2 mls 5% PCA and vortex. Centrifuge @ 2,500 rpm in IEC tabletop centrifuge for 10 minutes. Remove supernatant and save
2. Resuspend pellet in 2 mls 5% PCA and vortex. Centrifuge as above. Remove supernatant and save
3. Resuspend pellet in 2 mls 50% MeOH and vortex. Centrifuge as above. Remove supernatant and save
4. Resuspend pellet in 2 mls 50% MeOH and vortex. Centrifuge as above. Remove supernatant and save.
5. Resuspend pellet in 2 mls 1:1 Et₂O/EtOH and vortex. Cover with parafilm. centrifuge as above. Remove supernatant and save.
6. Resuspend pellet in 2 mls 1:1 Et₂O/EtOH and vortex. Cover with parafilm. centrifuge as above. Remove supernatant and save.
7. Resuspend pellet in 0.5 ml of 0.1 M potassium hydroxide and incubate overnight at 37° C
8. Sample should be clear by the next day. Neutralize to pH 7.0 with hydrochloric acid. Determine approximate volume with pipetman.

#3 Bradford Protein Concentration Assay

Reagents:

- Bradford Protein Assay Reagent (Pierce)
- bovine serum albumin(BSA)-2mg/ml stock solution in water (Sigma, St. Louis, MO)

Procedure:

Standard concentration prepared from 2 mg/ml stock

<u>Concentration</u>	<u>Preparation</u>
1.0 mg/ml	0.5 ml stock + 0.5 ml water
0.5 mg/ml	0.5 ml of 1.0 mg/ml + 0.5 ml water
0.25 mg/ml	0.5 ml of 0.5 mg/ml + 0.5 ml water
0.1 mg/ml	0.1 ml of 1.0 mg/ml + 0.9 ml water
0	water

--all samples run in duplicate

--0.05 ml of BSA solution added to 0.05 mls of Bradford Protein Assay Reagent and A₅₉₅ measured on Shimadzu 2101PC spectrophotometer.

--0.05 ml of each unknown sample added to 0.05 ml of Bradford Protein Assay Reagent and incubated for 5-90mins. A₅₉₅ measured on Shimadzu 2101PC spectrophotometer. Concentration determined against the standard curve

APPENDIX III

Calculations

Total Protein Binding

AMS

$$\begin{aligned} & \text{(Fraction Modern (measured))} \times (6.1 \text{ fCi } ^{14}\text{C/mg carbon}) \\ & \quad \times (\text{fmol } ^{14}\text{C-HQ}/22.2 \text{ fCi}) \\ & \quad \times (10^{-6} \text{ nmol/fmol}) \\ & \quad = \text{nmol } ^{14}\text{C-HQ/mg Carbon} \\ & (\text{nmol } ^{14}\text{C-HQ/mg Carbon}) \times (0.15 \text{ mg C}/1.0 \text{ mg protein}) \\ & \quad = \text{nmol } ^{14}\text{C-HQ/mg protein} \end{aligned}$$

Scintillation Counting

$$\begin{aligned} & \frac{\text{DPM}}{\text{volume (ml)}} \times \frac{\mu\text{curie}}{2.22 \times 10^6 \text{ DPM}} \times \frac{\mu\text{mole (}^{14}\text{)C-HQ}}{22.2 \mu\text{curie}} \times \frac{\text{ml of sample}}{\text{mg protein}} \\ & = \frac{\mu\text{mole (}^{14}\text{)C-HQ}}{\mu\text{gram protein}} \times \frac{1000 \mu\text{gram protein}}{\text{milligram protein}} = \frac{\mu\text{mole (}^{14}\text{)C-HQ}}{\text{mg protein}} \end{aligned}$$

1-D PAGE Analysis

For each segment measured:

$$\begin{aligned} & \text{Fraction Modern(measured)} \times \frac{97.9 \text{ fmol } ^{14}\text{C/g carbon}}{\text{Fraction Modern}} \times \frac{1}{\mu\text{gram protein}} . \\ & = \frac{\text{femtomole } ^{14}\text{C}}{\mu\text{gram protein}} \end{aligned}$$

APPENDIX IV

Abbreviations and Definitions

Abbreviations:

1-D PAGE: One dimensional gel electrophoresis

2-D PAGE: Two dimensional gel electrophoresis

Definitions:

PCA: Perchloric acid

ppm: Parts of the substance per million parts of air. This is a measure of the concentration in air.

STEL: Short Term Exposure Limit. This is a standard set for all chemicals which is the maximum level of exposure which workers may be exposed for 15 minutes only.

TWA: Time Weighted Average. This refers to the time-weighted average concentration over an 8 hour day and a 40 hour work week.