

Differential Effects of Organic and Inorganic Mercury on Phenotypically Variant Breast Cancer Cell Lines

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Abstract

Mercury remains a major environmental contaminant world-wide. Increasing evidence supports heavy metals as estrogenic agents (metalloestrogens), yet the impact of organic and inorganic mercury is unclear. The intent of this study was to increase understanding of mercury compounds as tumor causing agents and their modes of action. Changes in cellular viability, proliferation, and apoptosis after exposure to inorganic (HgCl_2) and organic (CH_3Hg^+) mercury, were examined using a control human breast epithelial cell line (MCF-12A) compared to the human breast cancer cell lines, MCF-7 (ER+) and MDA-MB-453 (ER-). Exposure to mercuric compounds increased cellular proliferation (2-fold), altered caspase activity and p53 expression in a compound dependent manner in MCF-12A cells. Between the two tumor lines, MDA-MB-453 cells appeared most like MCF-12A cells with regard to the mercury response. MCF-7 (ER+) cells on the other hand, were relatively resistant to the effect of both HgCl_2 and CH_3Hg^+ as indicated by the relative lack of change in any of the parameters measured. The proliferation and viability of MCF-7 cells was not significantly affected and although 5 ppm CH_3Hg^+ significantly increased caspase activity, there was a net inhibition of p53 expression at the same concentration. Collectively, MCF-12A (normal epithelial) cells were most sensitive to both HgCl_2 and CH_3Hg^+ . Cellular adaptation and activation of the p53/apoptosis pathway would minimize tumorigenesis which was dependent on the chemical form of mercury. These protective effects were not observed in the tumor cell lines with the MCF-7 (ER+) cells being the most resistant to the mercury effects. A significant difference between MCF-7 and MDA-MB-453 cells is expression of the estrogen receptor, suggesting potential involvement of this receptor in the effects of mercuric compounds. Further work is necessary to examine the potential role of the estrogen receptor in modulating the effects of mercuric compounds on tumor development.

Keywords Estrogen receptor; p53; Caspase; Mercuric chloride; Methyl mercury; Apoptosis

Introduction

Heavy metals such as cadmium, cobalt and lead among others are considered to be “metalloestrogens”, which interfere with, or disrupt, the estrogen system and its subsequent cellular pathways. Metalloestrogens can be found in cosmetics, pesticides, food additives, tobacco smoke, industrial operations and other environmental contaminants [1]. There has been a growing interest in these metalloestrogens and their role in estrogen-dependent breast cancers [2,3]. Evidence has suggested that each of the estrogen receptors (ER α , ER β and GPR30) can be involved in the development of various breast cancers [2-5]. There has been little research exploring the potential of mercury as a metalloestrogen and its effect on estrogen receptor mediated pathways. To be a metalloestrogen, the compound does not have to directly influence the functioning of estrogen receptors; instead, it may affect one or more steps in the estrogen-response cascade [6]. For those reasons we have chosen to examine inorganic and organic mercury effects on breast cancer cell line growth. The lines consist of a control epithelial line, an ER+ line and an ER- line.

The amount of mercury that is still used in commercial products would surprise some consumers [7]. Although exposure risk has been reduced over the last few decades, there are still multiple exposure routes. Environmental exposure to mercury compounds is varied.

Besides industrial exposure, the major means by which a person can be exposed is by consuming contaminated fish [8]. Major chemical forms of mercury are elemental, organic (methylmercury; CH_3Hg^+), or inorganic (mercuric chloride; HgCl_2). Of these, methylmercury appears to be the most pervasive with regard to ingestion, and inhalation exposures [8]. Once exposed, the most sensitive population appears to be the very young and the very old. Exposure via the ingestion of contaminated seafood is the best understood pathway [8-10]. Since the fetus or newborn represents a highly sensitive population to the actions of mercury, there is considerable concern that mercury can be passed to the newborn via the breast milk [11,12]. Contamination of consumer products can also be an area of concern and some imported ‘natural’ remedies have been shown to be contaminated with mercury [13]. Of particular interest is the ability of mercury to remain in the body for extended periods, bioaccumulating and posing a risk later in life.

Multiple organ systems are affected by mercury exposure [14,15]. One target for mercury toxicity is the enzyme-rich environment of the liver [16,17]. Mercury has been shown to readily bind to exposed thiol groups on enzymes, receptors and transporters as well as interfering with the proper functioning of hepatic P450 enzymes [17,18]. Although the liver is a major target organ, there is evidence that other organ/hormonal systems can also be targeted. These include kidney, heart, thyroid, reproductive system and brain [14,15,19-21]. Of particular interest is the ability of mercury compounds to interfere with immune/inflammatory responses as well as elicit genotoxic

responses in cells [14,15,22]. Interference with DNA repair or pro-inflammatory responses may be the foundation for mercury involvement in the development of breast cancer. With the potential for metals to elicit estrogen-like responses, correlations between metal exposure/concentration have been sought [2,5,20]. Although not entirely clear, others have determined there are correlations between transitional heavy metals and the incidence of breast cancer [23-25]. The role of aluminum in the development of breast cancer, potentially involving antiperspirants and some cosmetics as factors [23,24,26]. Mercury can bind directly to estrogen receptors with high affinity and significantly reduce the total number of estrogen binding sites [27,28]. The reduction in estrogen binding sites is not due to an alteration in the DNA binding domain since estrogen will still bind with nanomolar affinity when reconstituted with mercury in the binding domain. This is an effect not observed with other metals such as manganese, nickel and lead [29]. This lack of DNA binding domain effect is similar to what was observed with cadmium, suggesting that mercury and cadmium may share some metalloestrogen effects [29]. Increasing evidence now suggests that mercury will interfere with the estrogen system, either at the receptor level, or intracellular signaling level which may lead to the development of breast cancers where the estrogen receptor may be vital for pathogenesis.

Not all breast cancers require a functional estrogen receptor. Therefore, additional investigation is needed into the intracellular effects of mercury, to examine non-estrogen-dependent effects, on breast cancer cells. One potential target for mercury action is through the apoptotic signaling pathways. This would involve function/activity of caspase enzymes and/or the tumor suppressor protein, p53. The family of cysteine-aspartic acid protease (caspase) enzymes consists of multiple isozymes involved in the apoptotic pathway. For the current study, we chose to examine the activity of caspase 3 and caspase 7. The advantage to examining these caspase enzymes is that they recognize the Asp-x-x-Asp tetrapeptide motif [30]. Caspase isozymes exist as an inactive proenzyme that must be modified for activation by extrinsic (death ligand), intrinsic (mitochondrial) pathways or both [31,32]. The inactive precursor is cleaved by another initiator caspase (extrinsic) which prevents unchecked cell death caused by caspase 3. Once activated, the catalytic site of caspase 3 involves the sulfhydryl group of Cys-285 and the imidazole ring of His-237. The importance of this is the ability of mercury to bind to thiol groups, potentially interfering with the Cys-285 group, and thus altering the activity of caspase. Therefore we intend to examine not only the cellular changes in caspase 3 and 7 activity, but to examine the effects of both organic and inorganic mercury on purified caspase 3 and caspase 7 activity.

The tumor suppressor p53 is a protein encoded by TP53 (humans) and Trp53 (mice) and has a very important role in maintaining the normal functioning of a cell. Activity of this protein will permit arresting the cell cycle resulting in damage repair, or elimination if the damage is not repairable via an apoptotic pathway. Therefore p53 is pivotal for normal cellular function and is one of the most frequently mutated genes in the development of cancer [33]. There are multiple domains on the p53 molecule where mutations or mercury interactions can take place [34]. Activation domain 2 (amino acids 43-63) is responsible for the regulation of proapoptotic genes, the central DNA-binding core domain (containing a zinc atom), and the homo-oligomerization domain (amino acids 307-355) which is responsible for p53 tetramerization that is vital for full p53 activity. Each region has the potential for multiple mercury interactive sites.

Many reviews have discussed the actions of heavy metals as potential carcinogens and in most instances; these metals can also fall within the metalloestrogen category [35,36]. Cell death is due in part to the activation of apoptotic pathways. For example, arsenic has been shown to downregulate the Ube2d gene [37], downregulate bcl-2 and p53 [35] and promote the formation of reactive oxygen species [35]. Other divalent metals have been shown to alter the expression of multiple nuclear transcription factors (AP-1, NF- κ B, p53, NFAT and HIF-1) which are responsible for the protection of the cell through regulation of DNA repair, cell cycle function and apoptosis [36]. Both organic and inorganic mercury have been shown to damage a variety of cell types. In lung epithelial cells, methylmercury induces damage via oxidative stress as well as an upregulation of pro-apoptotic proteins [38]. Hepatocytes exposed to mercuric chloride were not subjected to apoptotic cell death, but instead were induced to autophagic death with a parallel increase in p53 expression [39]. Beta cells in the pancreas are very sensitive to the damaging effects of both organic and inorganic mercury [40]. In addition to oxidative stress, downregulation of bcl-2, mcl-1 and mdm2 in conjunction with an upregulation of p53 and caspase 3 and 7 expression suggests multiple modes of cellular damage [40].

Low concentrations of mercury may affect apoptosis in cells through both intrinsic and extrinsic pathways further substantiating the potential importance of mercury as a breast cancer causing agent. Of interest in this study are low-concentration effects on the function of phenotypically different breast cancer cell lines (MCF-7; MCF-12A and MDA-MB-453). The MCF-7 cell line (Michigan Cancer Foundation) has been heavily studied under numerous assay conditions [21,26-28,41-44]. MCF-7 cells are one of the classic estrogen receptor expressing (ER+) breast cancer cell lines and substantial work has been done to characterize estrogen receptor responsiveness in these cells [27,28,41,42]. Recently, investigations into the effects of mercury on cellular function in MCF-7 cells, and the potential estrogenic effects of mercury, have suggested that mercury may exert estrogen-like effects [27,28,42]. Since MCF-7 cells are used as ER+ breast tumor cells, appropriate control cells need to be utilized. In many instances, the control cells used are normal epithelial breast tissue cells, MCF-12A [45,46]. Similar to MCF-7 cells, the MCF-12A line also expresses ER α as well as GPR30 making it an excellent estrogenic control cell line [46]. In many studies, the MCF-12A cell line is used as the epithelial control line to the ER+ MCF-7 cell line [47-51]. The third cell line, MDA-MB-453 (M.D. Anderson), does not express estrogen receptors or progesterin receptors, but does express androgen receptors and HER2/ERK-1/2 [52]. The MDA-MB-453 line has been used to examine unique cellular pathways that may be functional in non-classic breast cancers [53-55]. Activation of androgen receptors on MDA-MB-453 cells results in cellular proliferation that is blocked by progesterin, through an antagonistic action on the androgen receptor [56]. These data will be the first to describe the action of mercury on MDA-MB-453 cells since to date; there have been no reports in the literature regarding these actions.

It is hypothesized that organic and inorganic mercury will alter cellular function in 3 breast cancer cell lines in both a chemical form X cell line dependent manner. Each mercury compound has multiple sites of action some distinct between chemical forms, but also some overlap. Although improbable to test 'all' breast cancer cell lines, we have chosen 3 lines based on their phenotypical variations. Through direct and/or indirect mechanisms, mercury will alter the function of these cells through oxidative stress or interactions with apoptotic systems as indicated by caspase 3 and 7 activity and p53 expression.

We also hypothesize a marked difference in sensitivity would be observed in cell lines with varying expression of estrogen receptors which may be due to the reported estrogenic properties of mercury.

Materials and Methods

Cell Cultures

All cell lines are obtained from ATCC. MCF-7 (ATCC® HTB-22™); MCF-12A (ATCC® CRL-10782™) and MDA-MB-453 (ATCC® HTB-131™) are grown according to the suggested protocol from the manufacturer. Table 1 below compares the cells types with regard to ERα/β, progesterone, HER2/neu activity and p53 activity. Prior to the exposure, all cultures will be grown in their normal assigned growth media. MCF7 cells were cultured in Dulbecco's modified Eagles MEM with 10% FBS, 4 mM L-glutamine, 1% Insulin transferrin and 1% penicillin/streptomycin. MCF12 cells were cultured in DMEM/F12K mixture (1:1) supplemented with 5% horse serum, human epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), insulin transferrin

(0.01mg/ml) and hydrocortisone (500 ng/ml). All cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere. MDA-MB-453 cells were cultured in Leibovitz L-15 with 10% FBS and no CO₂ incubation at 37°C. To standardize all assay procedures during exposure, each of the cultures will be grown in DMEM without phenol red, 1% FBS (low FBS) and without 1% penicillin/streptomycin (cytotoxicity media). The use of the cytotoxicity media reduces the chance that serum components or phenol red will interfere with the assay. All cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere (except for MDA-MB-453 in L-15 media). All media was obtained from American Type Culture Collection (ATCC; Manassas, VA), Sigma-Aldrich (St. Louis, MO), or Mediatech, Inc. (Corning; Manassas, VA). All media additions were obtained from either MediaTech, Inc. (Fetal bovine-FBS and Horse serum-HS), Sigma-Aldrich (penicillin/streptomycin, glutamine, hydrocortisone, human epidermal growth factor, cholera toxin), or Gibco/Life Technologies (Grand Island, NY; Insulin-Transferrin-Selenium). Both HgCl₂ and CH₃Hg⁺ were obtained from Sigma-Aldrich.

Cell Type	ERα Receptor	ERβ Receptor	Progesterone Receptor	HER2/neu	p53
MCF-7	[+]	[+]	[+]	[-]	[+] (WT)
MCF-12A	[+]	[-]	[-]	[+/-]	[+/-]
MDA-MB-453	[-]	[-]	[-]	[+]	[-]

[+]: Cell line expresses the protein; [-]: Cell line does not express the protein, or expresses at very low levels; [+/-]: Variable data that does not clearly define expression or non-expression.
(WT): Wild Type cell line; ERα/ERβ: Estrogen receptor α and β; HER2/neu: Human Epidermal growth factor Receptor 2.

Table 1: Comparison of key proteins expressed in each of the cell lines.

Metal interactions with pure LDH, caspase 3 or caspase 7 activity

Purified bovine heart L-Lactate Dehydrogenase (LDH; 1,000units/mL) was purchased from Sigma-Aldrich (St. Louis, MO) and purified lyophilized human caspase 3 (300,000 units/mg) and 7 (25,000 units/mg) were purchased from G Biosciences (St. Louis, MO). Each of the enzymes was then diluted to a final concentration of 1 unit/μl in phosphate-buffered saline (PBS). Aliquots were stored at -80°C until use. Enzymes were then diluted approximately 1:500 for use in inhibition studies for a final quantity of enzyme in each well equal to 0.2units/well. Stock concentrations of HgCl₂ and CH₃Hg⁺ were prepared in PBS at a concentration of 1000 ppm and diluted initially 1:100 for 10 ppm working concentration at the highest concentration and then 1:100 again for a working concentration of 0.1 ppm. Assays were then performed as described for the measurement of LDH activity as well as caspase 3 and 7 activity. All assays were performed in black/clear bottom 96-well plates.

Treatment of cell lines

Cells were plated at 105 cells/ml in the appropriate 96-well plate; clear for non-fluorescence and black/clear bottom for fluorescence assays, and returned to the incubator for 24 hours. Cells were then exposed to either methylmercury CH₃Hg⁺ (organic; 0.1, 0.5, 5 or 10 ppm), mercuric chloride HgCl₂ (inorganic; 0.1, 0.5, 5 or 10 ppm), or media alone (control) in 1% FBS DMEM without phenol red. This reduced FBS cytotoxicity media alleviates potential for false positives,

possible interaction with assay reagents and to equilibrate all media types for comparability across cell lines. Depending on the assay, the cells were allowed to incubate at 37°C in 5% CO₂ humidified atmosphere for 24 or 48 hours prior to analysis for cellular viability, proliferation, caspase 3/7 activity, p53 expression and oxidative stress. All stock solutions of CH₃Hg⁺ and HgCl₂ were prepared in sterilized distilled water, and diluted at least 1:100 for the highest concentration resulting in <1% dilution of the media components by the addition of mercury-containing assay media. The highest concentration used for each of the mercury compounds was 10 ppm which corresponds to 36.9 μM (HgCl₂) and 39.8 μM (CH₃Hg⁺).

Assessment of cellular viability: Media and Mercury effects

Each cell line has its unique growth media. Since comparisons were to be made between cell lines, the various components of the different media solutions would be unnecessary confounds for data interpretation. To reduce media variation as a potential data confounding factor and to reduce the risk for component of serum (or phenol red) to interfere with the assay, a "cytotoxicity media" was used. This media was DMEM, without phenol red and with no glutamine. Glutamine was added back to the media at a concentration of 4 mM and FBS was added to a final concentration of 1%. Cells did not experience adverse effects to the reduced serum in preliminary studies and the small quantity of serum was included to avoid issues associated with serum-deprivation. Cell of the cell lines were plated into black/clear 96-well plates with a density of 105 cells/ml and allowed to adhere for 24-48 hours. Growth media is removed, and the

cells were washed with 100 μ l of cytotoxicity media. After the wash is removed, another 100 μ l of warm cytotoxicity media is added to each of the wells. Cells were incubated in the cytotoxicity media for 48 hours at which time, cell viability studies were conducted with CytoTox-ONE™ (Promega; Madison, WI). The principle of this reaction is released LDH can be measured using the enzymatic reaction that results in the conversion of resazurin (non-fluorescent) into resorufin (fluorescent), which is directly proportional to the amount of LDH in the media. The CytoTox-ONE™ reagent can be used directly on a population of live and dead cells since the supplied substrate does not damage healthy cells. Assays were performed according to the manufacturer's suggested protocol. Briefly, half of the cells were lysed by the addition of 3 μ l of 0.9% Triton-X for 1 hour at 37°C. After lysis, LDH substrate was added to all wells (1:1 volume to volume) and the reaction was allowed to proceed at room temperature protected from light for 10 minutes. The reaction was terminated by adding 50 μ l a stop reagent (2.5% SDS solution). Fluorescence was then measured with a Bio-Tek® plate reader and KC4™ software at 530/25 nm (excitation)/590/25 nm (emission). Viability of cells was then calculated using the following formula:

$$\frac{[Total\ LDH] - [Media\ LDH]}{[Total\ LDH]} \times 100 = \% \text{ Viable}$$

With total LDH being the fluorescence in the lysed wells (containing live and dead cells) and media LDH being the fluorescence associated with the nonlysed wells (only dead cells).

Assessment of mercury effects was performed essentially as described above. Cells were plated in their respective growth media and allowed to adhere to the wells for 24 hours. Media was removed and wells washed once with warmed cytotoxicity media. Cytotoxicity media (100 μ l) was then added to each of the wells with the appropriate concentration of mercury compound. Cells were returned to the incubator and exposure was allowed to proceed for 24 or 48 hours depending on the experiment. Determination of LDH and calculation was viable cells was performed as described above.

Determination of cellular proliferation/growth

We were able to determine cellular proliferation/growth by subtracting the LDH released from the dead cells into the media, from the total LDH activity after complete cell lysis. Our concern was that we may see similar viability percentages across treatment groups, yet we may have significantly higher or lower cell counts within groups. The CytoTox-ONE™ assay kit can determine cell proliferation as well as viability. The mechanics of the assay are identical to the cellular viability assays and the data can be extracted from the same data sets avoiding the need to run different parallel assays, allowing for the direct comparison of percent viability and cellular proliferation. Contrary to other methods using tetrazolium salts which can be damaging to the cell itself, the use of the modified LDH assay permits the calculation of viability and proliferation/cell growth [57,58].

Measurement of apoptosis

Apoptosis, measured by caspase-3/7 activity, was determined using the Apo-One™ Homogeneous caspase-3/7 assay (Promega, Madison WI). The basic principle of the caspase 3/7 assay is that the assay buffer will allow cellular entry of the non-fluorescent substrate. The substrate rhodamine 110, bis-(N-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110), exists as a non-fluorescent substrate prior to the assay. Following cleavage and removal of the DEVD peptides

(recognized equally by caspase 3 and 7) the rhodamine 110 leaving group becomes intensely fluorescent at a wavelength of 499 nm. The emission maximum is 521 nm. The generation of the fluorescent product is then proportional to the amount of caspase 3/7 present. All cells were plated at a density of 105 cells/ml and were treated 24 after plating with the HgCl₂ (0.1, 0.5, 5 or 10 ppm) or CH₃Hg⁺ (0.1, 0.5, 5 or 10 ppm), in cytotoxicity media. The assay was carried out according to manufacturer's protocols. Briefly, after 48 hours of exposure, 100 μ l (1:1 with media) of the caspase 3/7 substrate/buffer mix was added to each of the wells and the plates were allowed to incubate at 37°C for 1 h. Fluorescence was then measured with a Bio-Tek® plate reader and KC4™ software at 485/25 nm (excitation)/530/25 nm (emission).

Measurement of p53 expression

The p53 production assay was conducted using the Pierce Colorimetric In-Cell ELISA kit. Each cell line was plated at a density of 104 cells/well in a clear 96-well plate. Cells were returned to the incubator and incubated overnight for 24 hours prior to treatment. Cells were treated according to the appropriate treatment groups of HgCl₂ or CH₃Hg⁺ for 48 hours. After exposure, the media was removed and 100 μ l of 4% formaldehyde was added to each well for 15 minutes at room temperature to fix the cells. The cells were washed 2X with 100 μ l/well of 1X Tris-buffered saline (TBS) followed by permeabilization buffer (100 μ l/well) for 15 minutes at room temperature. The cells were quenched and blocked with TBS washes at each step. Then 50 μ l/well of primary antibody (anti-p53; 1:1,000) was added, the plate sealed and was placed at 4°C overnight. The primary antibody solution was removed and the cells washed with TBS. Horseradish peroxidase (1:400) was added to the wells and allowed to incubate for 30 minutes at room temperature. TMB substrate (100 μ l/well) was added and the reaction was allowed to proceed for 15 minutes at room temperature, stopped by the addition of stop solution and absorbance read at 450 nm within 30 minutes of terminating the reaction. To correct/normalize for the number of cells present in each of the wells, whole cell staining was performed using Janus Green stain. Following a brief (5 minutes at room temperature) staining process and wash, the absorbance was measured at 615 nm. The amount of p53 expressed (absorbance at 450 nm) is divided by the total cell number stained by Janus Green (absorbance at 615 nm) which yields a normalized ratio of p53 expression per unit number of cells.

Measurement of oxidative stress by dichlorofluorescein (DCFH) fluorescence

Each of the cell lines were sub-cultured into black plates/clear bottoms at 104 cells/well. Each cell line was then returned to the incubator and allowed to adhere to the plates for 24 hours. After 24 hours, media was removed and cells were washed 2X with warm Krebs buffer. Stock DCFH in DMSO was diluted in pre-warmed Krebs to a final concentration of 100 μ M and 100 μ l was added to each well. Cells were returned to the incubator 30 min and after cellular loading of DCFH, the excess was removed and cells were washed with 2X with Krebs. Solutions of HgCl₂ and CH₃Hg⁺ [5 ppm] were prepared in Krebs buffer and each cell line received buffer only (control), 5 ppm HgCl₂ or 5 ppm CH₃Hg⁺. Plates are covered and allowed to incubate at room temperature for 30 minutes. After 30 minutes, generation of fluorescence was determined by using a Bio-Tek plate reader with the settings of 485 nm (excitation) and 585 nm (emission) with an integration time of 40 ms. Assays were run three separate times in

triplicate. Data analysis was first performed on the raw data, and then data was expressed as the mean ± SEM transformed to represent percent of control (buffer only) fluorescence.

Statistical analysis

Raw data prior to any transformation was analyzed with by one-way or two-way analysis of variance (ANOVA) where appropriate [One-way-Treatment effect; Two-way-Treatment X Time, or Concentration X treatment]. A significant ANOVA was followed by post hoc analysis using either Sidak's or Dunnett's test for multiple comparisons. If multiple comparisons were made between groups, not compared to control, then Sidak's test was used as a more powerful alternative to the Bonferroni test. Dunnett's test for multiple comparisons was performed when treatment groups were being compared to a control group (0 ppm) to determine at which time there was a deviance from the control mean. The Brown-Forsythe test was performed as part of the ANOVA to determine whether the standard deviation between groups was different. Analysis was completed if the Brown-Forsythe result was 'non-significant' meaning that the standard deviation between groups was not different. The threshold for statistical significance for all analyses was established with $\alpha = 0.05$.

Results

Effect of serum/media content on cellular viability and proliferation/cell growth

The responses to reduced serum were variable depending on the cell type. The assay was a 2 × 2 (Media X Time) design, and analyzed by 2-way ANOVA followed by Sidak's post-hoc comparisons (Table 2). In MCF-12A cells, phenol red-free and reduced (1%) FBS media significantly affected both cellular viability (F_{1,12}=4.85; p=0.0479) and proliferation (F_{1,12}=4.947; p=0.0461). Cell viability was significantly (p<0.05) reduced at 48 hours in the cytotoxicity media group (13%) compared to time-matched control, but overall, the viability ranged from 68-78%. Cell number was significantly (p<0.05) increased 29% in the 48 hour control group compared to control values. Differences in cell number were not observed between media groups until 48 hours where the cytotoxicity media group was reduced 23% compared to control values.

	Viability (%)				Cell Number (RFU)			
	Normal Media		Cytotox. Media		Normal Media		Cytotox. Media	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MCF-12A	71.96 ± 2.11	77.49 ± 0.95	75.83 ± 1.73	67.08c ± 0.68	5040.0 ± 196.1	6514.6a ± 320.5	5326.1 ± 403.3	4993.8c ± 66.1
MDA-MB-453	78.56 ± 0.35	87.03a ± 0.62	83.52b ± 0.46	84.53c ± 0.34	15248.1 ± 73.1	8081.3a ± 311.0	14881.8 ± 76.4	9851.3c,d ± 206.3
MCF-7	78.65 ± 1.10	73.63a ± 0.87	78.66 ± 0.33	74.77 ± 1.21	7146.0 ± 259.6	5341.1a ± 49.1	5779.3b ± 37.1	4113.4c,d ± 79.6

Normal growth media for each cell type is defined in methods, the cytotoxicity media is DMEM without phenol red, plus 1% (low) fetal bovine serum and 4 mM Glutamine. Data was analyzed by 2-way ANOVA (Media v. Time) comparison for each cell line. Post-hoc comparisons were completed using the Sidak's test with corrections for multiple comparisons. Data are expressed as mean ± S.E. for N=4 assayed in duplicate. A significance level was established at p<0.05.

a: 24 hour normal media v. 48 hour normal media
 b: 24 hour normal media v. 24 hour cytotoxicity media
 c: 48 hour normal media v. 48 hour cytotoxicity media
 d: 24 hour cytotoxicity media v. 48 hour cytotoxicity media

Table 2: Comparison of normal growth media and cytotoxicity assay media on cellular viability and cell number following 24-48 hour growth.

Changing the MDA-MB-453 growth media from Leibovitz L-15 in the absence of CO₂, to a robust media, with CO₂ and low FBS did produce significant changes in the viability and growth profile. Significant changes in viability were observed that were dependent on both time (F_{1,12}=104.2; p<0.0001) and media (F_{1,12}=6.179; p=0.0287). Interesting, viability was slightly (7%) higher in the cytotoxicity media at 24 hours compared to growth media controls. Proliferation of MDA-MB-453 was significantly lower at 48 hours compared to 24 hours in both the normal and cytotoxicity media groups (F_{1,12}=987.5; p<0.0001) as indicated by lower cell numbers. Normal media group cell number was 47% lower at 48 hours compared to 24 hours, and 33% lower in the cytotoxicity media group comparing the same time points suggesting a significant effect of media on the growth of MDA-MB-453 cells (F_{1,12}=13.08; p=0.0035). Uniformly, the

cytotoxicity media group was reduced compared to control growth media groups.

Surprisingly, MCF-7 cells exposed to organic or inorganic mercury did not exhibit the robust changes in cell viability and/or growth at either 24 or 48 hours as was observed in the other cell lines. There was no effect of media on cell viability (F_{1,12}=0.3752; p=0.552). Interestingly, viability was slightly elevated (p<0.05) at 48 hours compared to 24 hour values in both media groups. There were significant effects of both time (F_{1,12}=155.4; p<0.0001) and media (F_{1,12}=88.86; p<0.0001) on cell growth. Cell number was significantly (p<0.05) reduced at 48 hours in both the normal media and cytotoxicity media group (25% and 29% respectively) compared to 24 hour values. Comparison between media resulted in similar reductions. At 24 hours, the cytotoxicity media group was reduced 19% compared to control media and at 48 hours this differential was 23%.

Compared to the other cell lines, these changes were not as stout as those changes observed in the other cell lines.

Direct effect of metals on enzyme function

The ability of mercury to bind thiol groups on proteins was a concern with regard to the activity of caspase 3,7 and LDH. It was unknown as to whether mercury could potentially interact with these proteins, effecting enzyme function and thus, interfering with the interpretation of the results. It was apparent that neither CH_3Hg^+ nor HgCl_2 altered the activity ($F_{4,29}=2.61$; $p=0.056$) of purified caspase 3 or caspase 7 (Figure 1A). When examining the effects of CH_3Hg^+ or HgCl_2 on purified LDH activity (Figure 1B), exposure to both mercury compounds resulted in statistically significant ($F_{4,15}=26.45$; $p<0.0001$) elevations in LDH activity. Examining the increases, the actual increase in LDH activity observed in the treatment groups was relatively small, only 5-10% increase over control values, but each reached statistical significance following Dunnett's test for post-hoc comparison to control (0 ppm) values.

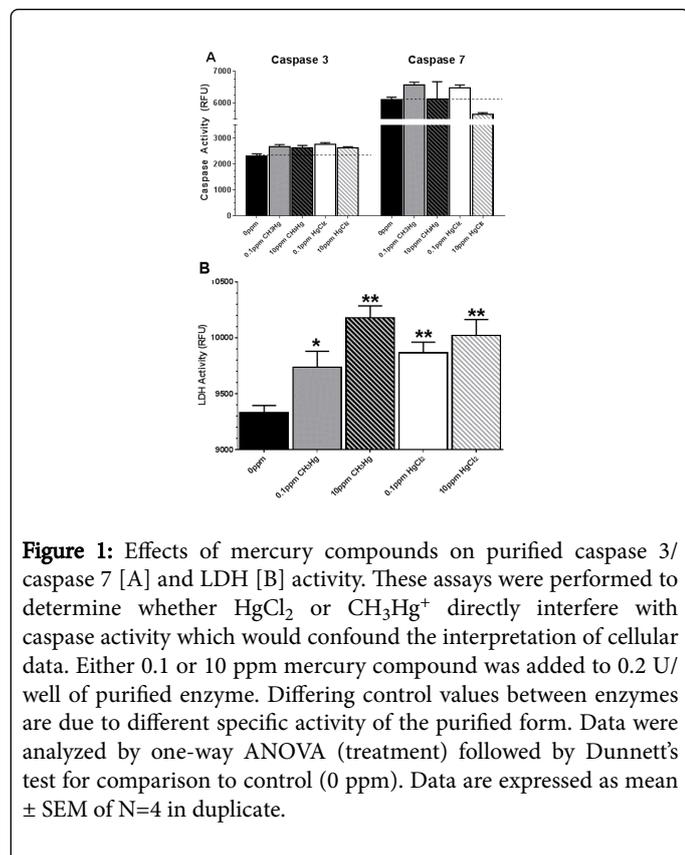


Figure 1: Effects of mercury compounds on purified caspase 3/7 [A] and LDH [B] activity. These assays were performed to determine whether HgCl_2 or CH_3Hg^+ directly interfere with caspase activity which would confound the interpretation of cellular data. Either 0.1 or 10 ppm mercury compound was added to 0.2 U/well of purified enzyme. Differing control values between enzymes are due to different specific activity of the purified form. Data were analyzed by one-way ANOVA (treatment) followed by Dunnett's test for comparison to control (0 ppm). Data are expressed as mean \pm SEM of N=4 in duplicate.

Effect of CH_3Hg^+ or HgCl_2 exposure on cell viability in breast cancer cells

MCF-12A (control), MDA-MB-453 and MCF-7 cells were exposed to either 0 ppm, 0.5 ppm or 5 ppm CH_3Hg^+ or HgCl_2 for 24 (Figure 2A) or 48 hours (Figure 2B) and viability was measured using the LDH assay. Following 24 hours of exposure (Figure 2A), mercury exposure resulted in significant alterations in LDH activity ($F_{4,45}=58.6$; $p<0.0001$). The control MCF-12A cells were sensitive to the effects of both CH_3Hg^+ and HgCl_2 , but only at the highest concentration of 5 ppm ($p<0.01$). MDA-MB-453 viability was significantly reduced only

at 5 ppm CH_3Hg^+ ($p<0.01$). MCF-7 cells were very sensitive to the effects of both CH_3Hg^+ and HgCl_2 , at all concentrations, but the viability was significantly ($p<0.01$) increased compared to control values. Following 48 hour exposure (Figure 2B), a similar profile was observed for both MCF-12A and MDA-MB-453 cells with the most pronounced reduction in viability being at 5 ppm CH_3Hg^+ with an increased reduction in viability at 5 ppm HgCl_2 . The lowest concentration of 0.5 ppm was relatively ineffective in both cell lines at both time points. Interestingly, MCF-7 cells exhibited a sensitivity to both 5 ppm CH_3Hg^+ and HgCl_2 at 48 hours with significant reductions ($p<0.01$) in viability observed. This was in contrast to the significant increases in cellular viability observed after 24 hour exposure.

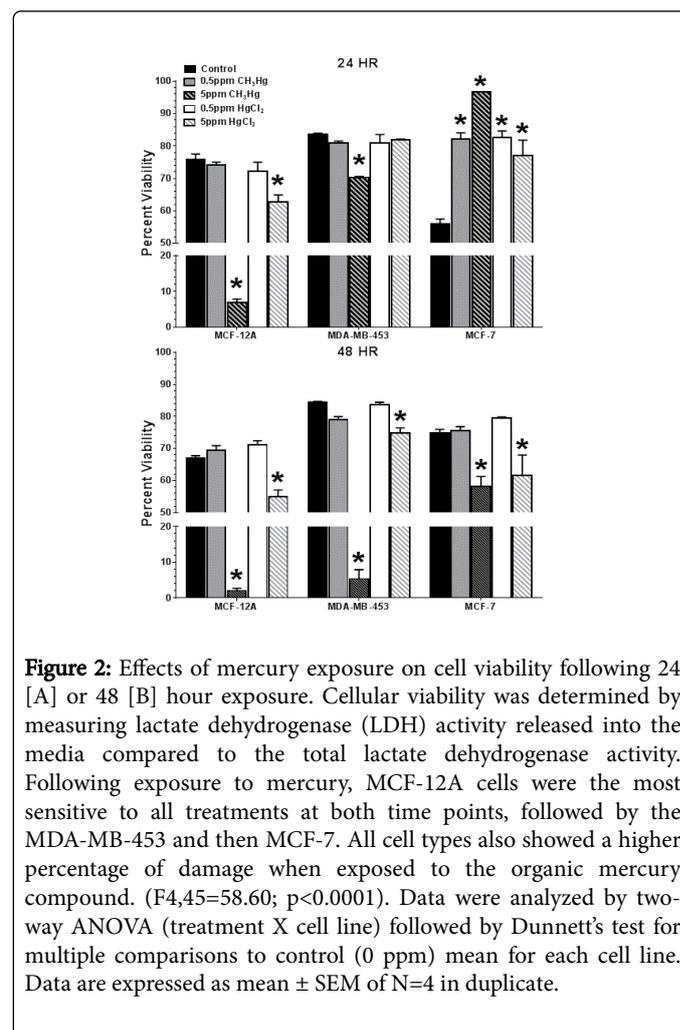


Figure 2: Effects of mercury exposure on cell viability following 24 [A] or 48 [B] hour exposure. Cellular viability was determined by measuring lactate dehydrogenase (LDH) activity released into the media compared to the total lactate dehydrogenase activity. Following exposure to mercury, MCF-12A cells were the most sensitive to all treatments at both time points, followed by the MDA-MB-453 and then MCF-7. All cell types also showed a higher percentage of damage when exposed to the organic mercury compound. ($F_{4,45}=58.60$; $p<0.0001$). Data were analyzed by two-way ANOVA (treatment X cell line) followed by Dunnett's test for multiple comparisons to control (0 ppm) mean for each cell line. Data are expressed as mean \pm SEM of N=4 in duplicate.

Effect of CH_3Hg^+ or HgCl_2 exposure on cell number/proliferation

MCF-12A (control), MDA-MB-453 and MCF-7 cells were exposed to either 0 ppm or 0.5 ppm CH_3Hg^+ or HgCl_2 for 48 hours (Figure 3) and the total number of viable cells was measured using the LDH assay. There was a significant difference dependent on cell type ($F_{2,27}=22.66$; $p<0.0001$) with MCF-12A cells exhibiting a significant ($p<0.01$) increase in viability after exposure to either CH_3Hg^+ or HgCl_2 . Both MDA-MB-453 and MCF-7 cells exhibited significantly ($p<0.01$) reduced proliferation following exposure to CH_3Hg^+ , whereas only MDA-MB-453 cell proliferation was reduced following exposure

to HgCl₂. The significant difference in treatment effect (F_{2,27}=165.3; p<0.0001) was reflected in the cell type-dependent effects on cellular proliferation.

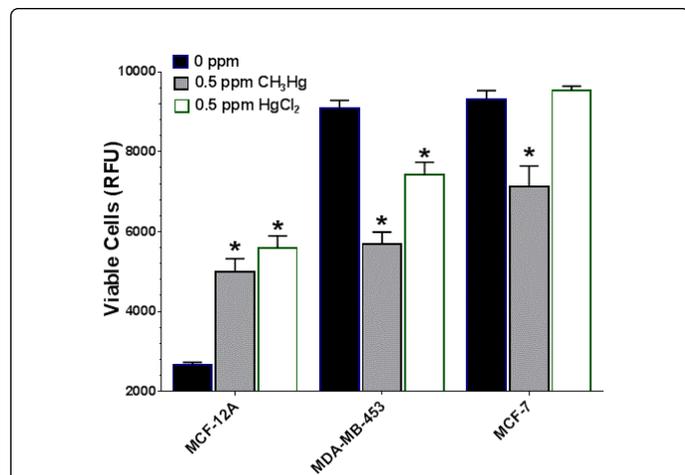


Figure 3: Effects of 48 hour mercury exposure (0.5 ppm) on cell number/proliferation. Relative cell number was determined by assessing the total number of live cells using lactate dehydrogenase (LDH) activity [Total cell LDH-Dead/Media LDH]. The concentration of 0.5 ppm was chosen based on its relative lack of effect on cell viability. Following exposure to mercury, MCF-12A cells were most sensitive to both CH₃Hg⁺ and HgCl₂ with significant increases (p<0.01) in cell number in both treatment groups. MDA-MB-453 cell number was significantly (p<0.01) reduced in both the CH₃Hg⁺ and HgCl₂ group compared to control. MCF-7 cells were the least sensitive, with only 0.5 ppm CH₃Hg⁺ group showing a reduction in cell number (p<0.01). Collectively, each of the cells appeared to be most sensitive to the organic form of mercury. Data were analyzed by one-way ANOVA (treatment X cell line) followed by Dunnett's test for multiple comparisons to control (0 ppm) mean for each cell line. Data are expressed as mean ± SEM of N=4 in duplicate.

Measurement of apoptosis

Exposure to 0.5 or 5 ppm CH₃Hg⁺ and HgCl₂ resulted in significant changes in caspase 3/7 activity (Figure 4) that were dependent on the cell line (F_{2,45}=7.64; p=0.0014). There was no significant effect of treatment as a whole (F_{4,45}=1.65; p=0.179). CH₃Hg⁺ exposure resulted in the most pronounced results, especially at 5 ppm where caspase 3/7 activity was significantly (p<0.01) reduced in both MCF-12A and MDA-MB-453 cells. MCF-7 cells responded in an inverse manner, with a significant (p<0.01) two-fold increase in caspase 3/7 activity compared to control values. Exposure to HgCl₂ resulted in a very different response profile. There was no effect in MCF-12A cells, with a significant (p<0.01) activity increase in MDA-MB-453 cells. Unlike MCF-7 response to CH₃Hg⁺, exposure to HgCl₂ resulted in a significant reduction in caspase 3/7 activity. Together, these data suggest that the chemical form of mercury is particularly important in determining the outcome regarding caspase 3/7 activity.

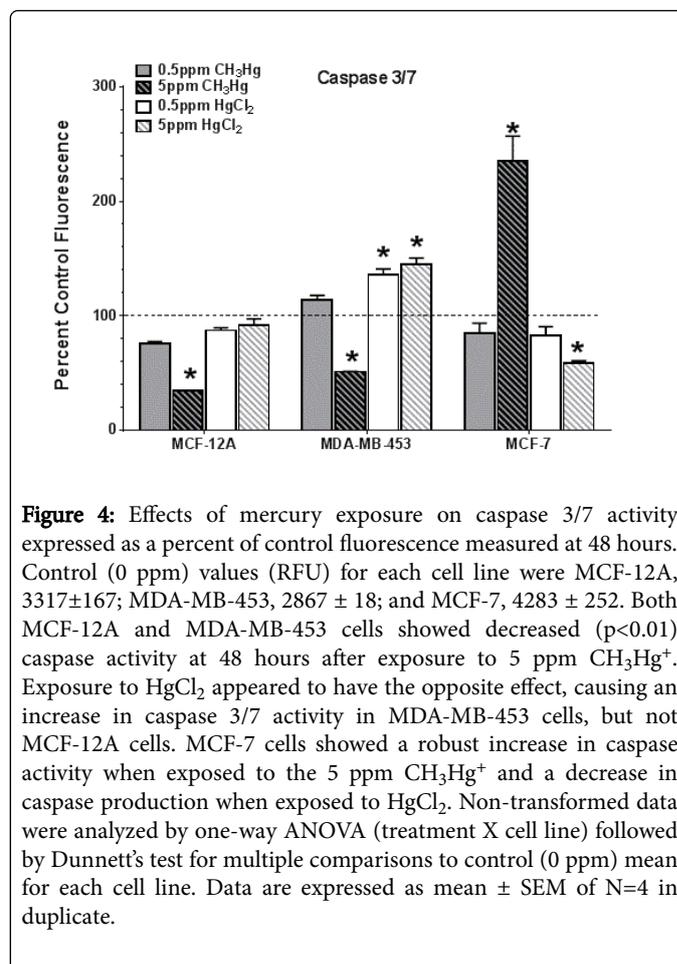


Figure 4: Effects of mercury exposure on caspase 3/7 activity expressed as a percent of control fluorescence measured at 48 hours. Control (0 ppm) values (RFU) for each cell line were MCF-12A, 3317±167; MDA-MB-453, 2867 ± 18; and MCF-7, 4283 ± 252. Both MCF-12A and MDA-MB-453 cells showed decreased (p<0.01) caspase activity at 48 hours after exposure to 5 ppm CH₃Hg⁺. Exposure to HgCl₂ appeared to have the opposite effect, causing an increase in caspase 3/7 activity in MDA-MB-453 cells, but not MCF-12A cells. MCF-7 cells showed a robust increase in caspase activity when exposed to the 5 ppm CH₃Hg⁺ and a decrease in caspase production when exposed to HgCl₂. Non-transformed data were analyzed by one-way ANOVA (treatment X cell line) followed by Dunnett's test for multiple comparisons to control (0 ppm) mean for each cell line. Data are expressed as mean ± SEM of N=4 in duplicate.

Measurement of p53 expression

Similar to the assay examining caspase 3/7 activity, cells were exposed to 0.5 ppm CH₃Hg⁺ or HgCl₂ for 48 hours and cells were fixed and probed for p53 expression, and viable cells were stained with Janus Green normalizing the p53 expression for differing cell numbers (Figure 5). The reason for the lower (0.5 ppm) concentration was that 5 ppm appeared to have maximum effects on viability, caspase activity, etc. Attempting to find more subtle changes, we exposed the cells to a lower concentration for 48 hours. There was a significant effect of treatment (F_{2,18}=15.48; p<0.0001) and cell type (F_{2,18}=19.96; p<0.0001). Post hoc comparison to control (0 ppm) values showed that MCF-12A cells exhibited a differential response with exposure to CH₃Hg⁺ resulting in a significant increase in p53 expression, yet exposure to HgCl₂ resulted in a significant (p<0.01) 50% reduction in p53 expression. Exposure to either CH₃Hg⁺ or HgCl₂ resulted in significant (p<0.01) reduction in p53 expression in MDA-MB-453 cells. Neither CH₃Hg⁺ nor HgCl₂ exposure altered p53 expression in MCF-7 cells.

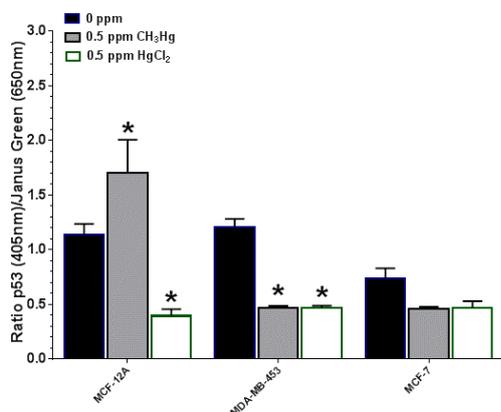


Figure 5: Determination of p53 expression following exposure to 0.5 ppm CH₃Hg⁺ or 0.5 ppm HgCl₂. MCF-12A cells exhibited the greatest increase in p53 expression following exposure to CH₃Hg⁺, yet exposure to HgCl₂ resulted in a net reduction in p53 expression suggesting that the chemical form of mercury is important for altering p53 expression (F_{2,18}=15.48; p<0.0001). In addition, cell type was a factor in the response to mercury (F_{2,18}=19.96; p<0.0001). In both MDA-MB-453 and MCF-7 cells, exposure to either the organic or inorganic form of mercury resulted in a reduction in p53 expression. These reductions only reached significance in the MDA-MB-453 cell line. Data were analyzed by one-way ANOVA (treatment X cell line) followed by Dunnett's test for multiple comparisons to control (0 ppm) mean for each cell line. Data are expressed as mean ± SEM of N=4 in duplicate.

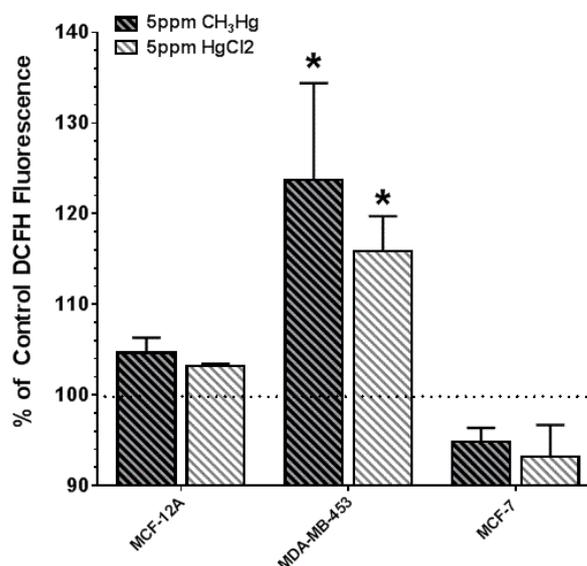


Figure 6: Oxidative stress was evaluated using the DCFH assay. MDA-MB-453 cells showed a significant deviation from control when exposed to both organic and inorganic mercury compounds. (F_{2,63}=7.1741; p=0.0016) Cell type was also observed to have a differential effect on the measured oxidative stress (F_{2,63}=349.9; p<0.001). Control values for baseline fluorescence (RFU) was MCF-12A, 371 ± 10; MDA-MB-453, 589 ± 37; MCF-7, 425 ± 11. Data (pre-transformation) were analyzed by one-way ANOVA (treatment X cell line) followed by Dunnett's test for multiple comparisons to control (0 ppm) mean for each cell line. Data are expressed as mean ± SEM of N=8 in duplicate.

Measurement of oxidative stress by dichlorofluorescein (dcfh) fluorescence

To determine the involvement of oxidative stress in mercury-induced toxicity, we examined the generation of fluorescence using dichlorofluorescein dye. Cells were loaded with 100 μM DCFH prior to exposure to 5 ppm CH₃Hg⁺ or HgCl₂. Fluorescence was then measured 30 minutes later (Figure 6). The drawback to this assay is that it will not assess long-term oxidative stress, or the potential for a toxin to reduce antioxidant enzymes following prolonged low-level oxidative stress. Responsiveness to mercury appeared to be more dependent on cell type (F_{2,63}=349.9; p<0.0001) than treatment (F_{2,63}=7.171; p=0.0016). The general trend was for CH₃Hg⁺ to elicit the larger response of the two treatments, but only in MDA-MB-453 cell was mercury-related alterations in DCFH fluorescence at statistical significance (p<0.01). MCF-12A cells demonstrated very small increases in DCFH fluorescence compared to control values, and MCF-7 cells exhibited small, non-significant, decreases.

Discussion and Conclusion

When comparing the literature on previous work in breast cancer cell lines, it is difficult to interpret and/or extrapolate across reports due to the multitude of cell lines, growth conditions and methodologies. One control that is frequently overlooked has been the ability of toxins such as heavy metals to interact directly with enzyme marker systems. We have shown that neither organic nor inorganic mercury interfere with caspase 3 or 7 activity and only a slight effect on purified LDH activity was observed. The LDH effect was in opposition to other reports that have suggested direct mercury interference with purified LDH resulting in a reduction in LDH activity [64,65]. Lack of interaction was our desired response to avoid potential confounding factors for data analysis and interpretation. Since the exposure data was compared to control values, any small effects of mercury on LDH activity would be accounted for and standardized. To avoid any confounding factors associated with the addition of phenol red to the media (free radical scavenging) or normal serum (>5% horse or fetal bovine) which can exert protective effects when exposed to toxins, we standardized the assay media to consist of standard DMEM, with the addition of 4 mM glutamine. After optimization, it was clear that control epithelial cells (MCF-12A) were most sensitive to time-dependent actions of both CH₃Hg⁺ and HgCl₂ with greater sensitivity displayed following exposure to organic mercury. Exposure to mercury

reduced viability of MCF-12A cells but did not affect the cellular growth as indicated by lack of variation in live cells. In fact, exposure to mercury (either organic or inorganic) resulted in a 2- to 3-fold increase in cell number after 48 hours. It was expected that some variations would be observed after switching/standardizing the media for cytotoxicity measurements. We chose the use of the resazurin-based assay for the ability to determine cellular viability and also determine cell number/proliferation. The MTT assay has long been used for cell viability measurements, but is limited in its usefulness [57,58]. The results seen with MDA-MB-453 cells were surprising in that switching to cytotoxicity media slightly increased cellular viability and proliferation at 48 hours compared to the normal media group at 48 hours. This is most likely due to switching from its growth media [L-15 with minimal supplementation and grown in a CO₂-free environment] to DMEM with more nutrients, but without phenol red and grown in 5% CO₂. Both MCF-12A and MCF-7 demonstrated minimal changes in viability up to 48 hours after switching the media. At 48 hours, both cell lines exhibited significant reductions in cellular proliferation/growth as indicated by reduced total number of live cells. In metal-toxicity studies, the use of 1% serum is optimum when examining either mercury or cadmium toxicity [59]. Other studies have shown that increasing the concentration of serum would increase cell number [60,61], which supports our findings that when serum content is reduced, cell number/proliferation is reduced.

Previous reports suggest mercury compounds impair cell viability and growth in multiple organ systems [37,38,40]. In breast cancer cell lines, the results have been anything but clear. In many instances, the effects observed are dependent on the type of growth media used, the chemical form of mercury, the duration of exposure, etc. We show mercury effects on cellular viability were dependent on time with greatest effects observed at 48 hours compared to 24 hours of exposure. In general, sensitivity was greatest to CH₃Hg⁺ with a rank-order of sensitivity being MCF-12A>MDA-MB-453>MCF-7. These responses were similar to other reports for mercury effects in pancreatic islet cells [40], kidney proximal tubule cells [37,63] and alveolar epithelial cells [38]. Although MCF-7 cells are better described than MCF-12A and MDA-MB-453, there has been minimal work describing the actions of mercury on these cell lines. These data are the first to describe the actions of mercury on MDA-MB-453 cells. In MCF-12A cells, only Schmidt et al. [45] reported that exposure to mercury reduces cell viability and proliferation after 4 days of exposure. Their work on lower concentrations demonstrates a clear increase in cellular viability and proliferation in MCF-12A cells, an effect not observed in tumorigenic cells, but supporting our current findings [45]. Their exposure time was 2-fold higher than our studies and their assays were performed with complete growth media for MCF-12A cells, not reduced serum. These media and methodology differences could explain some discrepancies in the time course for effects described in our study compared to their previous work. Proliferative effects of estrogen by mercury were proposed to be through direct action and expression of ERα [27,28,42]. Increased proliferation required 6 days of incubation with HgCl₂ with lesser exposure times not differing from control [27,28]. A modified assay media was used, phenol red-free, but only reducing serum to 5%, and not the 1% utilized in our studies. A report by Sukocheva et al. [42] suggests that the presence of estradiol is important for the mercury-induced increase in proliferation. When they used serum-free media, or a stripped-media that had any endogenous estradiol removed, there was no increase in proliferation. This supports our findings that in 1% FBS media, no increase in proliferation was observed and this may be due to lack of endogenous

estradiol present. Collectively these data suggest that the effects of mercury on breast cancer cells will be dependent on the chemical form of mercury as well as the type of breast cancer cell. Further examination is needed to determine whether the proliferation response is only due to ERα, or possibly ERβ (not expressed by MCF-12A or MDA-MB-453) which would explain the similarity between MCF-12A/MDA-MB-453 responses and their disparity with the MCF-7 response.

One potential mechanism underlying changes in cell growth is alterations in apoptotic pathways. Caspase 3/7 activity was chosen as the apoptotic marker since it is a common point for both intrinsic and/or extrinsic activity of the caspase cascade. Both caspase 8 (extrinsic) and caspase 9 (intrinsic) can regulate caspase 3/7 activity by promoting the cleavage and activation of the enzyme from the procaspase form. Since there are multiple potential points of action for mercury, we believe that this would be one of the best steps to examine and that alteration of caspase 8 and/or 9 activity would lead to changes in caspase 3/7 activity. Reductions in caspase 3/7 activity after exposure to 5 ppm CH₃Hg⁺ is counter to other reports following mercury exposure in different cell types [37,38,40]. In low-serum media, the induction of apoptosis in MCF-7 cells using CH₃Hg⁺ up to 20 μM resulting in approximately 0-20% apoptosis [42]. Others have used higher (up to 50 μM mercury) concentrations, and our highest at 5 ppm is approximately 20 μM. In MCF-7 cells, there was a CH₃Hg⁺-related increase in caspase 3/7 activity suggesting a caspase-dependent induction of apoptosis in these cells. An opposite (reduction) effect was observed following exposure to 5 ppm HgCl₂. Induction of caspase activity would be a cellular attempt to fix a damaged cell whereas a CH₃Hg⁺-induced reduction would promote tumorigenic growth and not cellular repair and normal growth. Collectively, these data suggest that both caspase-independent and caspase-dependent apoptotic pathways may be involved in cellular response to both CH₃Hg⁺ and HgCl₂. Reduced caspase 3/7 activity after CH₃Hg⁺ exposure would suggest an inhibition of the intrinsic and/or extrinsic pathway for caspase-mediated apoptosis. Additional studies would be necessary to determine if CH₃Hg⁺ is interacting with a cell surface binding site affecting the intracellular caspase cascade, or potentially inhibiting caspase 8 or 9 upstream from caspase 3/7 activation.

The data suggest that apoptosis is at least a partial factor in mercury-mediated effects in MCF-12A cells. Cells which under-express or lack a functional p21/p53 can have reduced cell numbers and survival [62] which may partly explain the difference cell line responses. Exposure to either CH₃Hg⁺ or HgCl₂ resulted in an interesting shift in p53 expression. In control MCF-12A cells, the increase in p53 expression was similar to what has been reported in other control cells expressing p53 [38,40]. Yet, others have reported that mercury-induced apoptosis functions via a non-p53-mediated mechanism, and that mercury exposure does not alter, or slightly reduces p53 expression [35,37,66]. One hypothesis is that mercury works through p38 and inhibition of NFκB to regulate apoptosis [35]. Clearly, mercury-related effects on p53 expression or activity are complicated and depend on the cell type. Using a different proapoptotic agent, Fickova et al. [66] showed in MCF-7 cells that p53 expression is unchanged and Bcl expression is reduced after 48 hours of exposure. This finding supports our findings that neither CH₃Hg⁺ nor HgCl₂ increased p53 expression in tumorigenic cells. In fact, in MDA-MB-453 cells, there was a significant reduction in p53 expression after exposure to CH₃Hg⁺ or HgCl₂. In control cells (such as MCF-12A) CH₃Hg⁺ induces p53 expression, suggesting proapoptotic actions at least partially dependent on p53. Exposure to

HgCl₂ resulted in a reduction in p53 expression, suggesting either an antiapoptotic mechanism, or a non-p53-related effect. In our studies, there is a clear 2-fold increase in p53 expression, with additional work necessary to examine how this elevation in p53 may relate to changes in Bax or p21CIP. It was clear that CH₃Hg⁺ and HgCl₂ exert markedly different effects in MCF-12A cells with opposing effects observed for caspase and p53 response, suggesting that the chemical form of mercury or possibly other heavy metals would be important in predicting the toxic outcome. Our findings begin to demonstrate that p53-mediated apoptosis in tumorigenic cells is either nonfunctional or negatively impacted by mercury exposure. Loss of apoptosis could result in increased migratory behavior or invasiveness of the tumor cells [26]. One conclusion is the mercury response is chemical form-dependent regarding whether the effects are mediated via a caspase-dependent pathway or a caspase-independent pathway. Obviously much more work needs to be done to further understand mercury involvement in apoptotic pathways in various breast cancer cells.

Collectively examining apoptotic data from the caspase and p53 assays, it appears that MDA-MB-453 cells were more like MCF-12A cells than the MCF-7 cells. In fact, mercury exposure increased the viability of MCF-7 cells at 24 hours, an effect which diminished by 48 hours. In neither cell line did we observe increased proliferation, instead, the general trend was for reduced cell number or no change compared to control values. The more dramatic changes were seen in the apoptosis measurements. MDA-MB-453 cells were more sensitive than MCF-7, but the profiles in response to organic v. inorganic mercury were quite different. Changes in caspase 3/7 activity were dependent on the chemical structure of mercury and in both cell lines; there was a reduced expression of p53 suggesting that these tumor cell lines may have blunted apoptotic responses to mercury. Lack of p53-mediated apoptosis may promote increased aggressiveness and invasiveness of the tumor cells [26]. Another mechanism utilized by mercury is alteration of mitochondrial membrane permeability. It has been shown that tamoxifen is toxic to cells by promoting increased permeability, leading to increased oxidative stress and cell death [41]. In 'normal' cells, mercury elicits a similar effect of increased mitochondrial permeability which is blocked by co-administration of tamoxifen suggesting a potential mitochondrial site of action for mercury [21].

The ability of mercury to generate reactive oxygen species/free radicals has been addressed many times, with no clear unequivocal results [67]. The ability to generate reactive oxygen species directly needs to be addressed. In vivo studies have shown evidence of reactive oxygen species generation by observing carbonyl formation, lipid peroxidation and a reduction in reactive oxygen species scavenging enzymes [40,67]. Similar results have been reported in vitro [35,38]. There is evidence that both organic and inorganic mercury alter mitochondrial function leading to increased membrane permeability, increased opening of transition pores and an increase in calcium leakage into the cytosol [21,67]. Our findings suggest that only MDA-MB-453 cells are affected by mercury exposure with both organic and inorganic mercury groups displaying significantly higher fluorescence levels compared to control. This increase in reactive oxygen species/oxidative stress production in a tumorigenic cell compared to the control MCF-12A cells has been shown before [51]. MCF-7 cells exposed to CH₃Hg⁺ have demonstrated elevated intracellular calcium levels, potentially leading to increased protein phosphorylation [42]. We conclude that in our current assay, reactive oxygen species are not a factor in the cytotoxicity (necrotic or apoptotic) observed in the cell-or treatment-specific manner.

Collectively, these data suggest that phenotypically variant breast cancer cell lines respond differently to different chemical forms of mercury. Others have reported similar findings following exposure to cytotoxic antiviral agents, MCF-12A cells displayed greater sensitivity compared to MCF-7 cells [47]. Control cells (non-tumorigenic) responded by attempting to enter apoptosis through increased expression of p53, a cellular response attempting to repair cellular damage to avoid becoming tumorigenic. The phenotypically different breast cancer cells displayed sensitivity responses (MDA-MB-453>MCF-7) suggesting a blunted cell death/apoptosis response. Exposure to mercury may then 'unmask' the ability of tumorigenic cells to proliferate and metastasize. These data support the need for additional work into characterizing different phenotypes of breast cancer by more established biomarkers [68] and to better established biological markers following exposure to heavy metals [69]. Further work is necessary to dissect the apoptotic pathway to more accurately determine where the difference chemical forms of mercury are acting, and what determines specificity of action. For example, if cellular proliferation is effected, more specific analysis of the point (G0,G1,G2,S, and M phase) at which cell growth/division is altered needs to be investigated. The current assay methodology only permits the determination of the number of live cells. Also, if the cells ability to enter apoptosis is affected, the question that needs to be asked is: "which stage of the apoptosis cascade is affected?" The present studies provide a foundation to move forward investigating the potential role of heavy metals/metalloestrogens in the development of breast cancer specifically or cancers in general.

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