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GENE EXPRESSION PROFILING OF HUMAN LIVER CARCINOMA (HepG2) CELLS EXPOSED TO THE MARINE TOXIN OKADAIC ACID

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Abstract

The marine toxin, okadaic acid (OA) is produced by dinoflagellates of the genera *Prorocentrum* and *Dinophysis* and is the causative agent of the syndrome known as diarrhetic shellfish poisoning (DSP). In addition, OA acts as both a tumor promoter, attributed to OA-induced inhibition of protein phosphatases as well as an inducer of apoptosis. To better understand the potentially divergent toxicological profile of OA, the concentration dependent cytotoxicity and alterations in gene expression on the human liver tumor cell line HepG2 upon OA exposure were determined using RNA microarrays, DNA fragmentation, and cell proliferation assays as well as determinations of cell detachment and cell death in different concentrations of OA. mRNA expression was quantified for approximately 15,000 genes. Cell attachment and proliferation were both negatively correlated with OA concentration. Detached cells displayed necrotic DNA signatures but apoptosis also was broadly observed. Data suggest that OA has a concentration dependent effect on cell cycle, which might explain the divergent effects that at low concentration OA stimulates genes involved in the cell cycle and at high concentrations it stimulates apoptosis.

Introduction

Okadaic acid (OA) and the related dinophysistoxins (DTX) are phycotoxins produced by several marine dinoflagellates belonging to the genera *Prorocentrum* and *Dinophysis*. Dinoflagellates are a highly diverse group of flagellated, unicellular protists which are responsible for the majority of toxic harmful algal blooms (HAB). Filter feeding shellfish

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can accumulate the toxin and the consumption of OA-contaminated shellfish results in a syndrome known as diarrhetic shellfish poisoning (DSP) which is characterized by severe gastrointestinal symptoms (Reguera and Pizarro 2008). DSP has been associated with the consumption of mussels, scallops or clams tainted with OA and its derivatives. Outbreaks of DSP have been reported in the Americas, Asia and Europe (Reguera and Pizarro 2008; Swanson et al. 2010; Deeds et al. 2010). The effects of chronic exposure to OA may be of greater concern, as it has been demonstrated to be a potent tumor promoter that differs from the phorbol ester class (Fujiki and Suganuma 1993; 2009).

The adverse effects of OA exposure are varied and cell-type and concentration-dependent (Gehringer 2004). OA is a potent inhibitor of the serine/threonine protein phosphatases PP-1, PP-2A (Fernandez et al. 2002; Dawson and Holmes 1999), PP-4 and -5 (Zhang et al. 1994) and PP-6 (Prickett and Brautigam 2006). It has been suggested that most or all of the toxic effects of OA may be attributed to the resulting hyperphosphorylation of numerous proteins leading to a loss of regulation of multiple intracellular processes, including cell cycle regulation, maintenance of cell shape, cytoskeletal disruption, cell motility, transport of vesicles, and caveolae (Sheppeck et al. 1997; Lontay et al. 2005; Honkanen and Golden 2002; Kuroda et al. 2007; Botos et al. 2007; Kiss and Botos 2009; Vale and Botana 2008; Blankson et al. 1995; Jayaraj et al. 2009). The commercial availability of OA isolated from cultures of *Prorocentrum concavum* has led to its prominent role in cell biological research, where it is often employed to enhance the activity of protein serine/threonine kinases such as the microtubule associated protein (MAP) kinases (Gómez et al. 1990), mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated protein kinase (ERK) (Barford 1996) in studies of cell cycle, apoptosis, nitric oxide metabolism and calcium signaling.

OA may act both as a tumor promoter and inducer of apoptosis in its role as a phosphatase inhibitor, via activation or inactivation of intracellular signaling cascades that are dependent on the phosphorylation state of proteins. In primary cell cultures OA exposure resulted in hyperphosphorylation of both p53 and the retinoblastoma protein (Yatsunami et al. 1993). After acute OA exposure, hyperphosphorylation of p53 led to cell cycle arrest and apoptosis (Yan et al. 1997), while hyperphosphorylation of the retinoblastoma protein led to upregulation of genes involved in DNA replication and cell proliferation (Gehringer 2004). Which of these opposite effects predominates in different human cell types has begun to be addressed (Valdiglesias et al. 2010; 2011a, 2011b, 2011c).

A growing body of evidence suggests that OA and its metabolites produced in the liver exhibit genotoxic effects (Fessard et al. 1996; Tohda et al. 1993; Pinto-Silva et al. 2005; Carvalho et al. 2003; Le Hegarat et al. 2003, 2004; Valdiglesias et al. 2010). The dependence of these effects on specific phosphorylation events has not been studied. OA exerts toxic effects independent of phosphatase inhibition, producing oxygen free radicals that impair protein synthesis (Matias et al. 1999).

In mice, OA was distributed equally to all organs but appeared to have the longest residence time in liver (Matias et al. 1996, 1999; Ito et al. 2002). The aim of this investigation was (1) to expand these studies and those of Valdiglesias et al. (2010, 2011a, 2011b, 2011c) in the human liver tumor model, HepG2, (2) examine the gene expression profile of exposure to OA and (3) correlate effects on cell cycle and apoptosis with concentration.

MATERIALS AND METHODS

Preparation of okadaic acid (OA)

Okadaic acid used in this study was isolated from cultures of *Prorocentrum hoffmanianum* according to published methods (Hu et al. 1992). The isolated OA was dried to a constant weight, quantitated gravimetrically and compared by HPLC-MS to OA that was purchased from Calbiochem (San Diego, CA). Isolated OA was found to be >96% pure by HPLC-MS and identical to commercial OA.

HepG2 cell culture and assessment of cell response to OA

HepG2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in normal media consisting of Eagle's Minimal Essential Medium (ATCC) with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 100 U/ml penicillin plus 100 µg/ml streptomycin (Gibco) in a 5% CO₂ humidified atmosphere at 37°C. Cells were passaged via trypsinization (0.05%; CellGro, Mediatech, Inc., Manassas, VA) into vented T25 flasks (BD Biosciences, Bedford, MA). Cells for an experiment were plated from the same mixture of donor cells at 1×10^6 cells in 5 ml media for bromodeoxyuridine (BrdU), RNA or DNA extraction experiments. Time zero counts for each flask (T₀), representing the number of cells just before toxin addition, were calculated approximately 18 hr after plating by averaging the cells counted in 3×1.08 mm² grids along the midline of the flask's long dimension and scaling up to the 25 cm² area of the flask. Following T₀ cell counts OA was added via substitution of 1 ml of media with 1 ml of a 5 fold working stock in media from a serially diluted stock of 620 µM OA in media. Calculated initial OA concentrations in triplicate flasks were 6.2, 41, 62, 206 or 620 nM. Media in control flasks was substituted with 1 ml of normal media without OA. Numbering of control and OA-exposed flasks was randomized by a colleague not involved in the study and assessments of cell morphology and cell numbers were made without knowing the OA concentration before breaking the code at the conclusion of experiments.

Cell morphological changes such as process retraction and cell rounding, and granular appearance suggesting impending cell death were assessed by inspection of cultures at 200× and scored as either present, if noted in >10% of attached cells, or absent, if present in <10% of attached cells. Cell detachment was graded by focusing the microscope objective on the surface of the media where these cells accumulated. Detachment was scored as absent or present at either a low or high extent based on this observation, as well as on cell counts (see T_{final}, below) and cell morphological assessments.

T_{final} (T_f) cell counts, representing the end of toxin exposure, were made on live cells after 48 hr exposure to OA according to the method described above. Cell-free spent media was frozen at -20°C for quantitation of OA and OA metabolites. The cells were harvested for either RNA or DNA extraction, or the cells adherent to the flask were fixed for BrdU assays (see below).

BrdU assay

After 24 hr incubation with OA, HepG2 cells were exposed to BrdU (10 µl/ml diluted from a concentrated stock solution of 10:1 5-bromo-2'-deoxyuridine:5-fluoro-2'-deoxyuridine according to the manufacturer's recommendations; Invitrogen). After an additional 24 hr, cells were fixed in the flasks with ethanol-glycine and T_f cell counts were made. BrdU positive cells were identified using a biotinylated antibody, mouse anti-BrdU (Calbiochem, San Diego, CA) and visualized using streptavidin-horseradish peroxidase and diaminobenzidine (DAB; Invitrogen, Camarillo, CA). Total cell numbers and % BrdU positive cells were determined by averaging cell numbers in 3×0.27 mm² grids along the

flask midline, then scaling to flask T_f cell numbers. Flasks with no BrdU and flasks with no primary antibody served as negative controls.

DNA Fragmentation Assay

After OA exposure, the media was removed, and HepG2 cells scraped from the substrate using a sterile cell scraper in 2 ml of digestion solution (50 mM Tris pH 8, 200 mM NaCl, 100 mM EDTA pH 8, 1% SDS, 0.2% DTT, 2 mg/ml Proteinase K). For exposures resulting in substantial cell detachment, cells were recovered from the culture media via centrifugation ($250 \times g$ for 8–10 min) and the resulting cell pellet added to scraped cells from the same flask. Samples were digested overnight at 55°C in a water bath. DNA was extracted from cells using standard phenol-chloroform extraction followed by precipitation in isopropanol. Purified DNA was resuspended in Tris EDTA and subsequently treated with 1–2 µl RNaseA (1mg/ml; Sigma-Aldrich), depending on volume of sample, for 20 min on ice to remove degraded RNA, then spectrophotometrically analyzed for concentration. Electrophoresis was performed on a 1.2% agarose gel at 80 V for 90 min. DNA gel-loading volumes were 2 µg of sample DNA per well for the 620 µM OA time course (Figure 2A) and 3 µg of sample DNA for cells exposed OA concentrations of 200–607 nM and analyzed as adherent vs detached cell fractions, to enhance ladder visualization (Figure 2B). DNA fragments were stained with GelRed (Biotium, Hayward, CA) and imaged using a Gel Doc imaging system (Bio-Rad) with Q capture imaging software.

Quantitation of OA and metabolite detection

One ml of each sample of spent media was extracted with methylene chloride (4×1 ml). The combined extracts were dried under a stream of N₂ and reconstituted in either 1 ml (highest 3 concentration samples) or 200 µl (control and lowest 2 concentration samples) of methanol. HPLC-MS analysis was carried out on a Finnigan Surveyor series HPLC system (Thermo Fisher Scientific Inc., Waltham, MA) using a 5 µm C18, 250 mm $\times$ 4.6 mm Discovery column (Supelco, Bellefonte, PA) coupled to a Finnigan LCQ Deca XP MAX mass ion-trap spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) as described previously (Guo et al. 2010). Ten µl of extract was injected. OA was quantitated against a single point calibration curve generated by injecting 10 µl of a 62 nM solution of OA. The instrument's limit of detection (LOD, defined as S/N = 3) was 0.1 ng for OA, and was presumed the same for OA metabolites. This detection limit was approached only in the case of the lowest concentration sample. OA concentrations presented in the Results and Discussion sections reflect those determined for spent media: 0, 2.4, 37, 51, 200 and 607 nM. Recovery of OA was determined by extraction from 0.5 ml of OA unused spiked media (125 nM and 6.2 nM OA concentrations) exactly as described above. Recoveries were calculated based on peak area compared to an OA standard. At 125 nM OA recovery was calculated to be 90%. However at 6.2 nM recovery was only 30%.

Functional assay for cytochrome P450 3A4 activity in HepG2 cells

CYP (cytochrome P 450) 3A4 activity was assayed according to methods of Yoshitomi et al. (2001) and Omasa et al. (2005). Four T75 flasks were plated with 4×10^6 cells each in 15 ml of culture media. A 100 mM stock solution of testosterone (Sigma-Aldrich Chemical) in methanol was diluted in media to give 4 15 $\times$ stock solutions (150 µM, 375 µM, 750 µM or 1.5 mM). One ml of culture media from each flask was replaced with 1 ml of testosterone containing media for final concentrations of 10, 25, 50 or 100 µM. Two ml aliquots of culture media were removed after 0.5, 1, 1.5 or 2 hr incubation for testosterone and 6 β -hydroxytestosterone analysis. Each 2 ml aliquot was extracted with ethyl acetate (3×2 ml). The combined extracts were evaporated in vacuo and reconstituted in methanol (1 ml). HPLC analysis was carried out on a Finnigan Surveyor series HPLC system (Thermo Fisher Scientific Inc., Waltham, MA) using a 5 µm C18, 250 mm $\times$ 4.6 mm Discovery column

(Supelco, Bellefonte, PA) and PDA (UV6000LP) detector monitoring at 244 nm. Twenty μl of sample was injected. The chromatographic conditions were as follows: flow rate of 1 ml/min; solvent A, CH_3CN ; solvent B, H_2O ; 0 – 5 min, 30% A; 5 – 10 min, 30–60% A; 10 – 20 min, 60% A; 20 – 25 min 60–90 % A. For additional confirmation, 10 μl of the 100 μM , 2 hr incubation sample was also analyzed using the more sensitive HPLC-MS on a Finnigan Surveyor series HPLC system (Thermo Fisher Scientific Inc., Waltham, MA) using a 5 μm C18, 250 mm $\times$ 4.6 mm Discovery column (Supelco, Bellefonte, PA) coupled to a Finnigan LCQ Deca XP MAX mass ion-trap spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) monitoring for m/z 288 $\pm$ 1 (testosterone) and m/z 304 $\pm$ 1 (6 β -hydroxytestosterone). The chromatographic conditions were identical to those described above. Control reactions (2 ml) were conducted by incubating testosterone (100 μM) with human recombinant CYP 3A4 (50 pmol, Codexis Corp.) or human liver microsomes (1 ml, 1 mg/ml, BD Biosciences). The reaction mixtures were extracted with ethyl acetate (3 $\times$ 2 ml). The combined extracts were evaporated in vacuo and reconstituted in methanol (1 ml) and analyzed by HPLC-PDA as described above. The instrument's limit of detection (LOD, defined as $S/N = 3$) was 2 ng for testosterone.

RNA Extraction and purification

After 48 hr exposure of HepG2 cells to OA or control solutions, culture media was removed from flasks and 2 ml chaotropic buffer (4.5M guanidinium thiocyanate, 2% N-lauroylsarcosine, 50 mM EDTA, 25 mM Tris-HCl, 0.1 M β -mercaptoethanol, and 0.2% antifoam A) was added. The cells were scraped off the bottom of the flask using a cell scraper, and removed by pipetting. At the two highest concentrations of OA some or all of the cells were detached. Spent media from these flasks was centrifuged at $250 \times g$ for 5 min and the cell pellet was added to the scraped cells from the same flask. RNA was isolated using standard protocols (Chomczynski and Sacchi 1987). Isolated RNA was purified using a Qiagen RNeasy Mini Kit (Valencia, CA) and quantitated using a Nanodrop spectrophotometer (ThermoFisher, Waltham, MA). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Microarray assay

Agilent G4112F Whole Human Genome 44K, Oligo microarrays were used to quantify mRNA expression. Hybridization used a loop design which is statistically superior to common reference design (Kerr and Churchill 2001; Churchill 2002) such that Control $\Rightarrow$ 37 nM $\Rightarrow$ 2.5 nM $\Rightarrow$ Control $\Rightarrow$ 37 nM $\Rightarrow$ 2.5 nM $\Rightarrow$ 51 nM $\Rightarrow$ 37 nM $\Rightarrow$ 200 nM $\Rightarrow$ 51 nM $\Rightarrow$ 607 nM $\Rightarrow$ 200 nM $\Rightarrow$ control $\Rightarrow$ 607 nM $\Rightarrow$ 200 nM $\Rightarrow$ control $\Rightarrow$ 37 nM $\Rightarrow$ 200 nM $\Rightarrow$ 607 nM $\Rightarrow$ 37 nM $\Rightarrow$ 2.5 nM $\Rightarrow$ 607 nM $\Rightarrow$ 2.5 nM $\Rightarrow$ 37 nM $\Rightarrow$ 51 nM $\Rightarrow$ 2.5 nM $\Rightarrow$ control $\Rightarrow$ 51 nM $\Rightarrow$ 51 nM $\Rightarrow$ control $\Rightarrow$ 2.5 nM $\Rightarrow$ 51 nM $\Rightarrow$ first sample (control); where each arrow represents an array with Cy3 labeled sample at its base and Cy5 labeled sample at the arrow head. Purified RNA was labeled using T7 linear amplification using a modified Eberwine protocol as described previously (Oleksiak et al. 2005; Whitehead and Crawford 2005, 2006a, 2006b; Crawford 2007; Scott et al. 2009). Two labeled reactions (Cy3 & Cy5) are re-suspended at 1.5 pmol/ μl and 2 $\mu\text{l}/\text{cm}^2$ (or 100 μl for each 50 cm^2 Agilent Array) of each reaction is used. Forty-eight hr hybridizations using Agilent reagents and protocols were employed. Following hybridization, slides were scanned using the Packard Bioscience ScanArray Express microarray scanner (PerkinElmer Life Sciences Inc., Boston, MA, USA) using a fixed power that maximizes signal without causing saturation. The resulting tiff images were imported into spot grids built in ImaGene (Biodiscovery Inc., Marina Del Ray, CA, USA), and spot signals were collected as fluorescence intensities for each dye channel.

Microarray data analysis and statistical treatment

Genes whose mRNA expression were below background or too highly expressed were excluded. Specifically, the fluorescent signals were considered to be too low if they were within 2 standard errors of background or too high if they saturated the photo-multiplier tube (i.e., equal to $2^{16}-1 = 65,535$). Of the 43,376 experimental probes, 15,037 were greater than background or not saturated (i.e., signal intensities were less than 65,535). Statistical analyses of the mRNA expression data were carried out using JMP genomics (SAS JMP Genomics v.7.0.2). All analyses used fluorescent measures that were log2 transformed and loess normalized.

An ANOVA of the 15,037 log2-loess measures (y_{ijk}) for the significant differences in gene expression among individuals within each group used the linear mixed model (Kerr and Churchill 2001; Wolfinger et al. 2001; Patterson et al. 2006; Yu et al. 2004)

$$y_{ijk} = \mu + A_i + D_j + T_m + \varepsilon_{ijk},$$

where μ is the sample mean, A_i is the effect of the i th array ($i=1-17$), D_j is the effect of the j th dye (Cy3 or Cy5), and T_m is one of 6 exposure to OA, and ε_{ijk} is the stochastic error. Each concentration was treated as a fixed effect, even though OA concentrations varied among replicates. With 6 treatments and 3 replicates per treatment the ANOVA used an F-table with 5 and 10 degree of freedom. The number of genes whose expression is significantly different across treatments (p-value of 0.01) without multiple correction is provided.

Dunnett's post-hoc test (JMP 7) was used to determine which concentrations differed from the control. "DAVID" at NCBI (Huang et al. 2009a, 2009b) was used to determine if functional categories (GO terms, metabolic pathways, protein structure function) were statistically over-represented. Over-represented is a statistical analysis and using a Chi-square analysis asks if the frequency of genes in a functional category is greater than expected given the data. Hierarchical clustering of gene expression uses Macintosh's version (de Hoon et al. 2004) of Eisen's Cluster and Treeview (Eisen et al. 1998). The criterion for significance was set at $p < 0.05$.

RESULTS

Cell proliferation, detachment and DNA integrity

Twenty-four hr exposure to OA resulted in marked morphological changes and detachment of HepG2 cells. Cells exposed to the highest concentrations of OA (200 or 607 nM based on concentrations in T_f medium) showed high cell detachment (>50%) from the substrate while cell detachment was less severe at 51 nM OA. However, at 51 nM OA, even cells remaining attached displayed rounded morphology suggesting cytoskeletal changes leading to impending detachment (data not shown). Fewer than 5% of cells were lost from the substrate in 37 nM OA at 24 hr but all cells were rounded, while in 2.5 nM no marked difference from control was noted in either attached cell numbers or in normal cell morphology. Deleterious changes were magnified after 48 hr exposure to OA, with both cell attachment and proliferation, as evidenced by BrdU staining, negatively correlated with OA (Figure 1A and B, respectively). Concentration response analyses resulted in slopes of 0.98 and 1.0 for attachment and proliferation, respectively, and half-inhibitory concentrations (EC_{50}) of 25.4 nM and 5.03 nM OA.

The DNA fragmentation assay for OA exposures to 200 nM OA demonstrated apoptosis and possible necrosis in adherent and detached cell fractions (Figure 2A and B). Apoptosis was indicated by the low molecular weight ladder (Figure 2A lanes 4–5; Figure 2B lanes 7–12) while necrosis was suggested by high molecular weight smear (lanes 8, 10). At each concentration in Figure 2B, detached fractions (lanes 8, 10, 12) appeared to have increased ladder formation relative to their adherent counterparts (lanes 7, 9, 11, respectively).

Cytochrome P450 activity in HepG2 cells

Guo et al. (2010) demonstrated that among 9 human CYP tested CYP3A4 and 3A5 metabolize OA to 4 metabolites. In order to ensure that all observed effects on HepG2 cells might be attributed to OA and not its metabolites, CYP 3A4 or 3A5 activity was assayed by incubation with testosterone. Incubation of HepG2 cells with testosterone produced no detectable 6 β -hydroxytestosterone, while control experiments using human recombinant CYP3A4 or human liver microsomes resulted in 70 and 60% conversion of testosterone respectively. HPLC-MS analysis of spent culture media indicated recoveries of OA ranging from 82 to 98% for the 4 most concentrated samples and as low as 39% for the least concentrated sample (Table 1). These recoveries are consistent with those observed from spiked media at similar concentrations. The poor recovery for the lowest concentration sample (HPLC-MS analysis of the original 6.2 nM sample was determined to be 2.4 nM) sample is more likely due to losses of OA at the lower concentration during the extraction process rather than metabolism of OA as none of the 4 previously characterized metabolites (Guo et al. 2010) were detected in any of the samples.

Gene expression analysis

Of the approximately 44,000 probes, only 15,037 were either less than saturating or displayed fluorescent data significantly greater than controls. Among these, 403 (2.7%) were significantly different across all 6 treatments (Table 2; Figure 3). Although 403 genes with significant differences in mRNA expression is 2.7 fold higher than the expected random frequency, only one of these is significant with Bonferroni's correction or 26 with FDR correction. Dunnett's test (a post-hoc test) indicates that 389 genes were significantly different between one of the treatments and the control (Figure 3), with the greatest number of significant genes at intermediate concentrations (37 and 51 nM, Table 2). Three-hundred and eighty-nine genes (2.6%) that were significant using the post-hoc Dunnett's test were 2.6 fold greater than the 1% random expectation. Our studies show that the expression of CYP RNAs was low and not significantly induced.

Regression analysis was performed on the 389 genes for which there was a significant effect on mRNA expression in the presence of OA. One hundred sixty nine genes had notable regressions. Specifically, 65 genes (17% of 389 genes with significant concentration effect) had a significant linear relationship using log₁₀ (Figure 4). One hundred fifty (39%) other genes had a non-linear response best fitted to a quadratic equation that significantly predicted a maximal response at intermediate concentrations (Figure 4). The more complex non-linear regression for these 150 patterns of mRNA expression was used because there was no significant linear regression. Data suggested that OA exerted complex effects on mRNA expression in this cell line. This complexity includes both log-linear and non-linear response to OA and thus the response to OA as measured by mRNA expression is not a simple dose response.

"DAVID", a program that uses NCBI GO terms, pathways and enzyme designations, was applied to determine if there were specific pathways or gene ontologies associated with either the significant difference relative to the control, or if there were concentration dependent effects. For both sets of significant genes (Dunnett's test comparing concentration

to control and regression) many more genes than expected by chance were involved in two pathways: cell cycle/cell replication or division and extracellular (secreted protein and extracellular matrix). The p-value indicated that genes in these pathways were greatly over-represented (Figure 4). Among these genes are several identified cell cycle related genes: CDKN2B (cyclin-dependent kinase inhibitor 2B), CCNA2 (cyclin 2A), ERBB3 (receptor tyrosine-protein kinase erbB-3), as well as apoptosis-related genes: CASP9 (caspase 9), CIDEB (cell death-inducing DFF45-like effector; DNA fragmentation factor) and DFFB (a caspase-activated DNase), and PERP (TP53 apoptosis effector). Several apoptosis-related genes, CASP9-related and ADAM17 (disintegrin and metalloprotease domain 17), were upregulated at intermediate concentrations of OA. Expression of AIF (apoptosis inducing factor), however, was not significantly altered at any concentration. CCNA2 was downregulated at low OA concentration while CDKN2 (cyclin-dependent kinase inhibitor 2) and CCNB2 (cyclin B2) were upregulated at low OA concentrations. ERBB3 was downregulated at high and intermediate OA concentrations. RBBP6 (retinoblastoma protein binding protein 6) was upregulated only at 200 μ M OA, whereas the RBR1 gene was not contained in this array.

DISCUSSION

Flanagan et al. (2001) reported a total loss of viability of HepG2 at 48 hr exposure to 1.24 μ M OA, producing changes prior to cell death that reflect cellular responses to toxic challenge such as alterations of the actin cytoskeleton. The studies presented here demonstrated toxic effects of OA at 50 nM. At OA concentrations below 25 nM for 48 hr, HepG2 cells proliferated at 50–80% the rate of controls, but at higher concentrations more negative effects of OA such as pronounced cell rounding and detachment were observed. These effects culminated in nearly complete cell detachment from the substrate at concentrations above approximately 50 nM OA in a process that included some apoptosis, as previously shown for OA (Valdiglesias et al 2011c; Fujiki and Suganuma 1993; Rossini et al. 2001). The similarity in slopes of the concentration-response relationships for detachment and proliferation suggest that cessation of proliferation and cell detachment are linked in a continuum of damage with increasing OA concentration. In addition, greater DNA laddering in detached fractions suggested greater apoptosis in detached cells.

The effects of OA on HepG2 cells appear to be independent of its metabolism via induction of phase 1 enzymes such as the CYP which is the route by which many carcinogens act (Perkinson 1996). CYP3A transforms several procarcinogens to their carcinogenic form (Medina-Diaz and Elizondo 2005; Perera 2010). Although Hashizume et al. (2009) demonstrated an increased tolerance to OA in HepG2 cells transformed to express CYP1A2. Guo et al. (2010) noted that among 9 human CYP tested including CYP1A2, only CYP3A4 and 3A5 metabolize OA. Therefore it is likely that OA did not induce CYP expression in HepG2 cells and OA toxicity in HepG2 cells may be attributed entirely to OA itself and not to its metabolites. Several lines of evidence lead us to this conclusion.

HepG2 cells exhibited low or undetectable levels of CYP3A4 or 3A5 activity in 2 studies (Yoshitomi et al. 2001; Omasa et al. 2005), with no CYP mRNA transcript levels detected in the former study and the latter study demonstrating only 0.6 pmol/min/mg testosterone 6 β hydroxylation by HepG2 cells. With the single exception of *o*, *p*-DDT (Medina-Diaz and Elizondo 2005), there is little evidence that most CYPs are more than minimally induced in HepG2 cells by the introduction of xenobiotics (Yoshitomi et al. 2001; Wilkening et al. 2003). OA was shown to be converted to 4 metabolites by human cytochrome P450 3A4 and 3A5 (Guo et al. 2010). However, incubation of OA with HepG2 cells failed to produce any of these metabolites. Testosterone metabolism, which is dependent on CYP3A4, was also not observed. Finally, mRNA expression of all members of the (CYP3A) family was low.

Valdiglesias and colleagues (2011a; 2011b; 2011c) studied OA toxicity in the range 0.005–1 μ M in different preparations including HepG2 cells to demonstrate differences in OA effects in different human cell types. Caspase 3-dependent apoptosis occurred in HepG2 cells exposed to OA for 3 hr without evidence of necrosis (Valdiglesias et al 2011c). A marker of γ H2AX phosphorylation denoting DNA damage in chromatin domains near DNA double strand breaks (Andrieviski and Wilkins 2009) was highest in HepG2 of the 3 cell types tested (Valdiglesias et al 2011b). In the comet assay, OA showed no marked oxidative damage to DNA in HepG2 cells (Valdiglesias et al 2011a). OA-induced DNA damage in HepG2 cells over a 48 hr exposure period was suggested by both apoptosis and necrosis in the detached cell fraction. DNA damage induced by CYP-dependent conversion of OA to its metabolites may not have been detected in these experiments due to absence of CYP expression in HepG2 cells. The lack of CYP expression is considered a shortcoming of the HepG2 cell line for genotoxicological testing studies. Guillouzo et al. (2007) developed a human liver-derived cell line, HepaRG, which is inducible for many CYP. It is possible that HepaRG might distinguish DNA damage induced by OA metabolites from the cell death induced by OA as documented here.

Several studies on gene expression in humans have demonstrated diverse actions of OA on cell processes. Chin et al (2000) used differential display in human glioma model T89G cells to show OA-induced upregulation of transcription factors, oxidative metabolism proteins, phosphorylation substrates, and stress response genes after 2.5 hr exposures. Valdiglesias et al (2012) used subtractive hybridization in the human neuroblastoma cell line SHSY5Y to show OA induced up and down gene regulation depending on the time of exposure from 2–48 hr with the affected genes playing roles in translation, transcription, signal transduction, metabolism, cell cycle control and apoptosis, transport, and cytoskeletal processes. The study of OA-mediated concentration effects adds to these insights. More genes were significantly different from the controls at OA concentrations of 37–200 nM than at low or high concentrations, with twice as many genes having curvilinear rather than linear responses. Expression of many genes peaked at intermediate concentrations. Most of these gene expression responses involved cell cycle and secretion or extracellular matrix genes. The array data showed specific cell cycle genes were downregulated at moderate OA concentrations consistent with the significant decrease in cell proliferation observed, while dozens more were upregulated at these same OA concentrations. Cell cycle inhibition at G0/G1 in HepG2 cells during short exposures to OA, with concomitant decreased expression of cyclins A2 and B1 and increased expression of cyclin D has been observed (Rubiolo et al. 2011; Valdiglesias et al. 2011c).

The upregulation of many array cell cycle genes may reflect the phosphorylation state of retinoblastoma protein, pRb, in the presence of OA, rather than a physiological response. pRb is a gatekeeper of cell cycle gene transcription that binds to the promoter region of cell cycle genes and whose activity is controlled by its phosphorylation state. pRb is active and in the promoter-bound form when dephosphorylated. When phosphorylated, it detaches from the promoter, allowing gene transcription. Since pRb is normally dephosphorylated by a serine/threonine phosphatase such as PP-1 or PP-2A (Magenta et al. 2008; Krucher et al. 2006; Grana 2008), in the presence of the PP-1 and PP-2A inhibitor OA, pRb would remain phosphorylated and inactivated, and cell cycle gene transcription would proceed. RBBP6 interacts with both pRb and p53 (Chibi et al. 2008), and promotes degradation of the latter, a sequence hypothesized to increase cell proliferation (Motadi et al. 2011). Thus OA may have induced inappropriate inactivation of both pRb and RBBP6, although it was possible to track only RBBP6 in our array, stimulating cell cycle gene transcription even though the cells were unable to divide due to damage and activation of apoptosis gene pathways. The observed gene expression patterns allude to both changes in morphology and the loss of viability in HepG2 cells.

In conclusion, the deleterious effects of OA on HepG2 cells reflect OA-induced toxicity and not that of its metabolites. While, as in past studies, many genes were affected by OA, cell cycle genes were significantly elevated at low OA while some apoptotic gene pathways were upregulated at moderate and high OA concentrations. Overall, the concentration dependent effects of OA on gene expression may explain the divergent effects of OA at low concentrations stimulating genes involved in the cell cycle while and at high concentrations stimulating apoptosis.

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References

- Andrievski A, Wilkins RC. The response of gamma-H2AX in human lymphocytes and lymphocytes subsets measured in whole blood cultures. *Int J Radiat Biol.* 2009; 85:369–376. [PubMed: 19399682]
- Barford D. Molecular mechanisms of the protein serine/threonine phosphatases. *Trends Biochem Sci.* 1996; 11:407–412. [PubMed: 8987393]
- Botos E, Turi A, Mullner N, Kovalszky I, Tatrai P, Kiss AL. Regulatory role of kinases and phosphatases on the internalization of caveolae in HepG2 cells. *Micron.* 2007; 38:313–320. [PubMed: 16713271]
- Blankson H, Holen I, Seglen PO. Disruption of the cytokeratin cytoskeleton and inhibition of hepatocytic autophagy by okadaic acid. *Exp Cell Res.* 1995; 218:522–530. [PubMed: 7540986]
- Carvalho Pinto-Silva CR, Ferreira JF, Costa RH, Belli Filho P, Creppy EE, Matias WG. Micronucleus induction in mussels exposed to okadaic acid. *Toxicol.* 2003; 41:93–97. [PubMed: 12467666]
- Chibi M, Meyer M, Skepu A, Rees G, DJ, Moolman-Smook JC, Pugh DJ. RBBP6 interacts with multifunctional protein YB-1 through its RING finger domain, leading to ubiquitination and proteosomal degradation of YB-1. *J Mol Biol.* 2008; 384:908–916. [PubMed: 18851979]
- Chin LS, Satyendra KS, Qant Q, Murray SF. Identification of okadaic acid-induced genes by mRNA differential display in glioma cells. *J Biomed Sci.* 2000; 7:152–159. [PubMed: 10754390]
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 1987; 162:156–159. [PubMed: 2440339]
- Churchill GA. Fundamentals of experimental design for cDNA microarrays. *Nat Genet.* 2002; 32:490–495. [PubMed: 12454643]
- Crawford DL. Human reference sequence makes sense of names. *Nature.* 2007; 447:142. [PubMed: 17495903]
- Dawson JF, Holmes CF. Molecular mechanisms underlying inhibition of protein phosphatases by marine toxins. *Front Biosci.* 1999; 4:D646–D658. [PubMed: 10502549]
- de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics.* 2004; 20:1453–1454. [PubMed: 14871861]
- Deeds JR, Wiles K, Heideman GB, White KD, Abraham A. First U.S. report of shellfish harvesting closures due to confirmed okadaic acid in Texas Gulf coast oysters. *Toxicol.* 2010; 55:1138–1146. [PubMed: 20060850]
- Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A.* 1998; 95:14863–14868. [PubMed: 9843981]
- Fernandez JJ, Candenias ML, Souto ML, Trujillo MM, Norte M. Okadaic acid, useful tool for studying cellular processes. *Curr Med Chem.* 2002; 9:229–262. [PubMed: 11860357]
- Fessard V, Grosse Y, Pfohl-Leszkowicz A, Puisieux-Dao S. Okadaic acid treatment induces DNA adduct formation in BHK21 C13 fibroblasts and HESV keratinocytes. *Mutat Res.* 1996; 361:133–141. [PubMed: 8980699]

- Flanagan AF, Callanan KR, Donlon J, Palmer R, Forde A, Kane M. A cytotoxicity assay for the detection and differentiation of two families of shellfish toxins. *Toxicon*. 2001; 39:1021–1027. [PubMed: 11223091]
- Fujiki H, Suganuma M. Carcinogenic aspects of protein phosphatase 1 and 2A inhibitors. *Prog Mol Subcell Biol*. 2009; 46:221–254. [PubMed: 19184590]
- Fujiki H, Suganuma M. Tumor promotion by inhibitors of protein phosphatases 1 and 2A: The okadaic acid class of compounds. *Adv Cancer Res*. 1993; 61:143–194. [PubMed: 8394044]
- Gehringer MM. Microcystin-LR and okadaic acid-induced cellular effects: A dualistic response. *FEBS Lett*. 2004; 557:1–8. [PubMed: 14741332]
- Gómez N, Tonks NK, Morrison C, Harmar T, Cohen P. Evidence for communication between nerve growth factor and protein tyrosine phosphorylation. *FEBS Lett*. 1990; 271:119–122. [PubMed: 2171996]
- Grana X. Downregulation of the phosphatase nuclear targeting subunit (PNUTS) triggers pRB dephosphorylation and apoptosis in pRB positive tumor cell lines. *Cancer Biol Ther*. 2008; 7:842–844. [PubMed: 18497566]
- Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F, Guguen-Guillouzo C. The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem-Biol Interact*. 2007; 168:66–73. [PubMed: 17241619]
- Guo F, An T, Rein KS. The algal hepatotoxin okadaic acid is a substrate for human cytochromes CYP3A4 and CYP3A5. *Toxicon*. 2010; 55:325–332. [PubMed: 19699225]
- Hashizume T, Yoshitomi S, Asahi S, Matsumura S, Chatani F, Oda H. In vitro micronucleus test in HepG2 transformants expressing a series of human cytochrome P450 isoforms with chemicals requiring metabolic activation. *Mutat Res*. 2009; 677:1–7. [PubMed: 19501186]
- Honkanen RE, Golden T. Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? *Curr Med Chem*. 2002; 9:2055–2075. [PubMed: 12369870]
- Hu T, Marr J, DeFreitas ASW, Quilliam MA, Walter JA, Wright JLC, Pleasance S. New diol esters isolated from cultures of the dinoflagellates *Prorocentrum lima* and *Prorocentrum concavum*. *J Nat Prod*. 1992; 55:1631–1637.
- Huang, da W.; Sherman, BT.; Lempicki, RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res*. 2009a; 37:1–13.
- Huang, da W.; Sherman, BT.; Lempicki, RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocol*. 2009b; 4:44–57.
- Ito E, Yasumoto T, Takai A, Imanishi S, Harada K. Investigation of the distribution and excretion of okadaic acid in mice using immunostaining method. *Toxicon*. 2002; 40:159–165. [PubMed: 11689237]
- Jayaraj R, Gupta N, Rao PV. Multiple signal transduction pathways in okadaic acid induced apoptosis in HeLa cells. *Toxicology*. 2009; 256:118–127. [PubMed: 19084044]
- Kerr MK, Churchill GA. Experimental design for gene expression microarrays. *Biostatistics*. 2001; 2:183–201. [PubMed: 12933549]
- Kiss AL, Botos E. Okadaic acid retains caveolae in multicaveolar clusters. *Pathol Oncol Res*. 2009; 15:479–486. [PubMed: 19067239]
- Kuroda F, Moss J, Vaughan M. Regulation of brefeldin A-inhibited guanine nucleotide-exchange protein 1 (BIG1) and BIG2 activity via PKA and protein phosphatase 1gamma. *Proc Natl Acad Sci U S A*. 2007; 104:3201–3206. [PubMed: 17360629]
- Krucher NA, Rubin E, Tedesco VC, Roberts MH, Sherry TC, De Leon G. Dephosphorylation of Rb (Thr-821) in response to cell stress. *Exp Cell Res*. 2006; 312:2757–2763. [PubMed: 16764854]
- Le Hegarat L, Fessard V, Poul JM, Dragacci S, Sanders P. Marine toxin okadaic acid induces aneuploidy in CHO-K1 cells in presence of rat liver postmitochondrial fraction, revealed by cytokinesis-block micronucleus assay coupled to FISH. *Environ Toxicol*. 2004; 19:123–128. [PubMed: 15037998]
- Le Hegarat L, Puech L, Fessard V, Poul JM, Dragacci S. Aneugenic potential of okadaic acid revealed by the micronucleus assay combined with the FISH technique in CHO-K1 cells. *Mutagenesis*. 2003; 18:293–298. [PubMed: 12714697]

- Lontay B, Kiss A, Gergely P, Hartshorne DJ, Erdodi F. Okadaic acid induces phosphorylation and translocation of myosin phosphatase target subunit 1 influencing myosin phosphorylation, stress fiber assembly and cell migration in HepG2 cells. *Cell Signal*. 2005; 17:1265–1275. [PubMed: 16038801]
- Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, Martelli F. Protein phosphatase 2A subunit PR70 interacts with pRb and mediates its dephosphorylation. *Mol Cell Biol*. 2008; 28:873–882. [PubMed: 17991896]
- Matias WG, Traore A, Bonini M, Sanni A, Creppy EE. Oxygen reactive radicals production in cell culture by okadaic acid and their implication in protein synthesis inhibition. *Human Exp Toxicol*. 1999; 18:634–639.
- Matias WG, Bonini M, Creppy EE. Inhibition of protein synthesis in a cell-free system and Vero cells by okadaic acid, a diarrhetic shellfish toxin. *J Toxicol Environ Health*. 1996; 48:309–317. [PubMed: 8656452]
- Medina-Diaz IM, Elizondo G. Transcriptional induction of CYP3A4 by o,p'-DDT in HepG2 cells. *Toxicol Lett*. 2005; 157:41–47. [PubMed: 15795092]
- Motadi LR, Bhoola KD, Dlamini Z. Expression and function of retinoblastoma binding protein 6 (RBBP6) in human lung cancer. *Immunobiology*. 2011; 216:1065–1073. [PubMed: 21676486]
- Oleksiak MF, Roach JL, Crawford DL. Natural variation in cardiac metabolism and gene expression in *Fundulus heteroclitus*. *Nat Genet*. 2005; 37:67–72. [PubMed: 15568023]
- Omasa T, Kim K, Hiramatsu S, Katakura Y, Kishimoto M, Enosawa S, Ohatake H. Construction and evaluation of drug-metabolizing cell line for bioartificial liver support system. *Biotechnol Prog*. 2005; 21:161–167. [PubMed: 15903254]
- Patterson TA, Lobenhofer EK, Fulmer-Smentek SB, Collins PJ, Chu T, Bao W, Fang H, Kawasaki ES, Hager J, Tikhonova IR, Walker SJ, Zhang L, Hurban P, de Longueville F, Fuscoe JC, Tong W, Shi L, Wolfinger RD. Performance comparison of one-color and two-color platforms within the Microarray Quality Control (MAQC) project. *Nat Biotechnol*. 2006; 24:1140–1150. [PubMed: 16964228]
- Perkinson, A. Biotransformation of xenobiotics. In: Klaassen, CD., editor. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. 5. New York: McGraw-Hill; 1996. p. 113–186.
- Perera M. The missing linkage: what pharmacogenetic associations are left to find in CYP3A? *Expert Opin Drug Metab Toxicol*. 2010; 6:17–28. [PubMed: 19968573]
- Pinto-Silva C, Creppy EE, Matias WG. Micronucleus test in mussels *Perna perna* fed with the toxic dinoflagellate *Prorocentrum lima*. *Arch Toxicol*. 2005; 79:422–426. [PubMed: 15692819]
- Prickett TD, Brautigan DL. The alpha4 regulatory subunit exerts opposing allosteric effects on protein phosphatases PP6 and PP2A. *J Biol Chem*. 2006; 28:30503–30511. [PubMed: 16895907]
- Reguera, B.; Pizarro, G. Planktonic dinoflagellates that contain polyether toxins of the old “DSP complex”. In: Botana, LM., editor. *Seafood and Freshwater Toxins*. 2. Boca Raton: CRC Press; 2008. p. 257–884.
- Rossini GP, Sgarbi N, Malaguti C. The toxic responses induced by okadaic acid involve processing of multiple caspase isoforms. *Toxicon*. 2001; 39:763–770. [PubMed: 11137534]
- Rubiolo JA, López-Alonso H, Vega FV, Vieytes MR, Botana LM. Okadaic acid and dinophys toxin 2 have differential toxicological effects in hepatic cell lines inducing cell cycle arrest, at G0/G1 or G2/M with aberrant mitosis depending on the cell line. *Arch Toxicol*. 2011; 85:1541–1550. [PubMed: 21512803]
- Scott CP, Williams DA, Crawford DL. The effect of genetic and environmental variation on metabolic gene expression. *Mol Ecol*. 2009; 18:2832–2843. [PubMed: 19500250]
- Sheppeck JE 2nd, Gauss CM, Chamberlin AR. Inhibition of the Ser-Thr phosphatases PP1 and PP2A by naturally occurring toxins. *Bioorg Med Chem*. 1997; 5:1739–1750. [PubMed: 9354230]
- Swanson KM, Flewelling LJ, Byrd M, Nunez A, Villareal TA. The 2008 Texas *Dinophysis* ovum bloom: distribution and toxicity. *Harmful Algae*. 2010; 9:190–199.
- Tohda H, Nagao M, Sugimura T, Oikawa A. Okadaic acid, a protein phosphatase inhibitor, induces sister-chromatid exchanges depending on the presence of bromodeoxyuridine. *Mutat Res*. 1993; 289:275–280. [PubMed: 7690896]

- Valdiglesias V, Fernández-Tajes J, Pásaro E, Méndez J, Laffon B. Identification of differentially expressed genes in SHSY5Y cells exposed to okadaic acid by suppression subtractive hybridization. *BMC Genomics*. 2012; 13:46–59. [PubMed: 22284234]
- Valdiglesias V, Laffon B, Pásaro E, Cemeli E, Anderson D, Méndez J. Induction of oxidative DNA damage by the marine toxin okadaic acid depends on human cell type. *Toxicol*. 2011a; 57:882–888. [PubMed: 21396392]
- Valdiglesias V, Laffon B, Pásaro E, Méndez J. Evaluation of okadaic acid-induced genotoxicity in human cells using the micronucleus test and aH2AX analysis. *J Toxicol Environ Health A*. 2011b; 74:980–992. [PubMed: 21707423]
- Valdiglesias V, Laffon B, Pásaro E, Méndez J. Okadaic acid induces morphological changes, apoptosis and cell cycle alterations in different human cell types. *J Environ Monit*. 2011c; 13:1831–1840. [PubMed: 21526234]
- Valdiglesias V, Méndez J, Pásaro E, Cemeli E, Anderson D, Laffon B. Assessment of okadaic acid effects on cytotoxicity, DNA damage and DNA repair in human cells. *Mutat Res*. 2010; 689:74–79. [PubMed: 20621797]
- Vale C, Botana LM. Marine toxins and the cytoskeleton: okadaic acid and dinophysistoxins. *FEBS J*. 2008; 275:6060–6066. [PubMed: 19016863]
- Whitehead A, Crawford DL. Variation in tissue-specific gene expression among natural populations. *Genome Biol*. 2005; 6:R13. [PubMed: 15693942]
- Whitehead A, Crawford DL. Variation within and among species in gene expression: raw material for evolution. *Mol Ecol*. 2006a; 15:1197–1211. [PubMed: 16626448]
- Whitehead A, Crawford DL. Neutral and adaptive variation in gene expression. *Proc Natl Acad Sci U S A*. 2006b; 103:5425–5430. [PubMed: 16567645]
- Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. *Drug Metab Dispos*. 2003; 31:1035–1042. [PubMed: 12867492]
- Wolfinger RD, Gibson G, Wolfinger ED, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS. Assessing gene significance from cDNA microarray expression data via mixed models. *J Comput Biol*. 2001; 8:625–637. [PubMed: 11747616]
- Yan Y, Shay JW, Wright WE, Mumby MC. Inhibition of protein phosphatase activity induces p53-dependent apoptosis in the absence of p53 transactivation. *J Biol Chem*. 1997; 272:15220–15226. [PubMed: 9182545]
- Yatsunami J, Komori A, Ohta T, Suganuma M, Fujiki H. Hyperphosphorylation of retinoblastoma protein and p53 by okadaic acid, a tumor promoter. *Cancer Res*. 1993; 53:239–241. [PubMed: 8417815]
- Yoshitomi S, Ikemoto K, Takahashi J, Miki H, Namba M, Asahi S. Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. *Toxicol in Vitro*. 2001; 15:245–256. [PubMed: 11377097]
- Yu X, Chu T, Gibson G, Wolfinger RD. A mixed model approach to identify yeast transcriptional regulatory motifs via microarray experiments. *Stat Appl Genet Mol Biol*. 2004; 3 :1544–1565.
- Zhang Z, Zhao S, Long F, Zhang L, Bai G, Shima H, Nagao M, Lee EY. A mutant of protein phosphatase-1 that exhibits altered toxin sensitivity. *J Biol Chem*. 1994; 269:16997–17000. [PubMed: 8006004]

Fig. 1. Dose-response relationship between [OA] in spent media and HepG2 cell parameters. A. Detachment from substrate after 48 hr in OA, with slope of 0.98 and (EC₅₀) of 25.4 nM. B. Normalized cell proliferation during the last 24 hr of 48 hr exposure to OA, with slope of 1.0 and (EC₅₀) of 5.03 nM. Fifty-four percent of control cells ("10e-10" M) proliferated in 24 hr.

Fig. 2.

Agarose gel of DNA fragmentation in HepG2 cells by OA. A. Time course of DNA fragmentation via 607 nM OA (total cells). B. DNA fragmentation in adherent vs. detached cell fractions after 48 hrs at the indicated [OA] from 200–607 nM.

Fig. 3. Pattern of significant gene expression in HepG2 cells exposed to OA. Relative level of mRNA expression for gene significantly different among OA concentrations (0=Control, 1=2.4, 2=37, 3=51, 4= 200 and 5=607 nM OA). **A.** All 389 genes that differ from control (Dunnett's test, $p < 0.01$). **B.** Fifty-two genes with significant differences in expression and associated with cell cycle or replication. **C.** Sixty-five genes with significant differences in expression and associated with extracellular matrix or secretion. **D.** Colorbar indicating fold changes.

Fig. 4.

Regression: OA dose *versus* mRNA levels in HepG2 cells. Four out of 169 significant regressions of $\log_{10}$ transformed OA empirically derived doses. **A & B.** Two of the 65 linear regressions. **C & D.** Two of the 150 non-linear regressions. Dashed gray line is the best fit (but non-significant) linear equation. Y-axis are the normalized $\log_2$ expression data and x-axis is the $\log_{10}$ OA doses.

Table 1

Quantitation of recovered OA *

Original OA concentration (nM)	OA detected in spent media (nM $\pm$ sd, n=3)	%OA recovered
0	0	-
6.2	2.4 $\pm$ 0.3	39
41	37 $\pm$ 7.0	90
62	51 $\pm$ 4.6	82
206	200 $\pm$ 16	97
620	607 $\pm$ 66	98

* Single ion monitoring was used to monitor for OA metabolites, but none were detected.

Table 2

Summary of Gene Expression

Treatment	number	% Total Genes
Total number of genes Quantified	15,037	
ANOVA Significant ($p < 0.01$)	403	2.7%
Total Dunnett's Significant ($p < 0.01$)	389	2.6%
% of Significant		
2.5 nM vs Control	75	19.3%
37 nM vs Control	212	54.5%
51 nM vs Control	150	38.6%
200 nM vs Control	110	28.3%
607 nM vs Control	50	12.9%
Regress of genes with significant Dunnett's	170	43.7%
if log sig	65	16.7%
if non-linear log sign	150	38.6%