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A Homogeneous Cell-Based Assay for Measurement of Endogenous PON1 Activity

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Abstract

PON1 is a high density lipoprotein-associated enzyme that plays an important role in organophosphate detoxification and prevention of atherosclerosis. Thus, there is significant interest in identifying nutritional and pharmacological enhancers of PON1 activity. In order to identify such compounds, we developed a rapid homogeneous assay to detect endogenous cell-associated PON1 activity. PON1 activity was measured by the simple addition of fluorogenic PON1 substrate DEPFMU to live Huh7 cells in media and monitoring change in fluorescence. A specific PON1 inhibitor, 2-hydroxyquinoline, was used to confirm that the observed activity was due to PON1. The assay was optimized and characterized with regard to time course, substrate and sodium chloride concentration, number of cells and tolerance to DMSO and serum. Aspirin, quercetin and simvastatin are compounds reported to increase PON1 expression. Consistent with the literature and western blot data, these compounds enhanced PON1 activity in this assay with comparable efficacies and potencies. A known toxic compound did not increase assay signal. This assay method also detected PON1 activity in normal hepatocytes. Thus, a novel, homogenous assay for detection of endogenous PON1 expression has been developed and is amenable to high throughput screening for the identification of small molecules that enhance PON1 expression.

Keywords

PON1; DEPFMU; aspirin; quercetin; simvastatin

Introduction

Paraoxonase 1 (PON1) got its name from its ability to hydrolyze the organophosphate paraoxon, which is the toxic metabolite of the insecticide parathion [1]. Initial interest in this serum enzyme was in its role as a detoxifying serum enzyme. In addition to providing protection from certain insecticides, PON1 has also been found to be able to hydrolyze certain organophosphate-class chemical weapons such as sarin [2]. Thus, there is significant interest in identifying therapeutics that may enhance PON1 activity in serum to provide protection from chemical weapon poisoning.

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Subsequent to early and continuing work on the detoxifying activities of PON1, more recent work has generated interest in its role in atherosclerosis. Oxidation of low density lipoproteins (LDL)¹ is a key step in the pathophysiology of atherosclerosis [3]. PON1 has been shown in various *in vitro* studies to inhibit the accumulation of lipid peroxides in LDL and isolated carotid lesions [4-6]. It has been well established that high density lipoprotein (HDL) plays a protective role against atherosclerosis [7]. PON1 has been demonstrated to reside almost exclusively on HDL particles in serum [8]. PON1 has been shown to be expressed on the cell surface of hepatocyte cell lines and requires HDL for secretion [9]. In addition to inhibition of LDL oxidation, there is evidence from other studies that PON1 can protect the HDL particle from oxidation and preserve its functional integrity [10]. Although the *in vivo* substrate for PON1 has not been identified, a theory has been proposed that PON1 removes oxidized lipid from LDL and HDL through hydrolysis [11]. Additional evidence for the role of PON1 in atherosclerosis comes from the PON1 knockout mouse that displayed no serum paraoxonase activity and enhanced susceptibility to atherosclerosis as well as organophosphate toxicity [12]. In complementary studies, transgenic mice that over-express PON1 have enhanced resistance to atherosclerosis [13-16]. In a recent large human study, the incidence of major cardiac events was approximately 3-fold lower in patients with the highest quartile of PON1 activity compared to patients with PON1 activity in the lowest quartile [17]. Thus, the animal data, human correlative data and the *in vitro* data generated to date suggest that therapeutics that enhance PON1 concentration and/or activity could reduce the risk of CHD or delay its onset.

There are several classes of known modulators of PON1 gene expression. Cholesterol lowering drugs known as statins have been reported to increase PON1 gene transcription and enhance serum PON1 activity in human studies [18-21]. Specifically, the statins simvastatin, pitavastatin, atorvastatin have all been reported to enhance PON1 gene transcription. Dietary polyphenols, including flavonoids such as quercetin, and resveratrol have been shown to activate PON1 gene transcription via activation of the aryl hydrocarbon receptor (AhR) [22-25]. Recent data has indicated that aspirin can significantly increase PON1 transcription and high doses of aspirin had a significant affect on serum PON1 activity in animal studies [26]. The mechanism for aspirin induction of PON1 transcription was shown to be through activation of the AhR. There are also substances that down-regulate PON1 expression. Pro-inflammatory cytokines IL-1 β , IL-6, and TNF α have been shown to down regulate PON1 gene expression in cell culture and when injected into hamsters [27-29].

The most frequently used substrates for measuring PON1 activity are paraoxon and phenyl acetate. Paraoxon has high selectivity for PON1 in serum since it has been demonstrated that PON1 is the only serum enzyme capable of significant hydrolysis of this substrate [12]. However, the turn-over rate is low resulting in low sensitivity to PON1 activity [30]. Phenyl acetate is one of the best substrates for PON1 in terms of generating a relatively high turn-over rate [30], but it is not selective for PON1. For both of these substrates, generation of product is followed by absorbance. Chromogenic and fluorogenic assays for the lactonase activity of paraoxonases have been reported [31]. These substrates can be used to measure serum PON1 lactonase activity. However, these substrates can be hydrolyzed by all three paraoxonases and therefore would lack selectivity for a PON1-specific cell-based assay. In addition, these substrates have not been demonstrated to be selective for paraoxonases in eukaryotic cell lysates.

¹Abbreviations used: LDL, low density lipoprotein; HDL, high density lipoprotein; CHD, coronary heart disease; AhR, aryl hydrocarbon receptor; DEPFMU, 7-diethyl-phospho-6, 8- difluoro-4-methylumbelliferyl; 2HQ, 2-hydroxyquinoline; DMSO, dimethyl sulfoxide; CDNB, 1-chloro, 2-4 dinitrobenzene; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline, RFU, relative fluorescence units

Currently, studies of PON1 gene expression and its modulation by cytokines or small molecules have employed several different techniques. For direct analysis of PON1 gene transcription in un-modified cells, Northern blots or quantitative RT-PCR has been used [22,32]. However, neither of these methods allow for rapid and inexpensive screening of test substances. Luciferase reporter assays using fragments of the PON1 promoter have also been used to characterize PON1 promoter elements and assess effects of transcriptional modifiers of PON1 [23,26,33]. Luciferase assays can be used in high throughput fashion to screen large libraries of compounds. However, these assays become relatively expensive when used for many data points and only detect transcriptional modifiers, not compounds with other mechanisms such as enhancing catalytic stability. In addition, artifacts are sometimes observed in cell-based reporter assays, such as was reported by Ota et. al. for the PON1 promoter [20]. In addition to western blot, others have reported methods to indirectly measure PON1 protein expression by assessing cell-associated and secreted PON1 activity. Secreted PON1 activity has been measured by transferring cell culture media to plates and adding typical substrates like paraoxon, phenyl acetate or p-nitrophenyl acetate to monitor catalysis [9,26]. However, most PON1 in cell culture cells appears to remain on the surface of the cell until actively transferred to HDL particles [9]. Thus, measuring secreted PON1 may not accurately reflect total PON1 made by the cell. Cell-associated PON1 activity has been measured by removing old media, adding substrate for an incubation period followed by removing supernatant from attached cells (or centrifuged cells) then measuring change in absorbance in a plate reader [9,26]. Thus, current methods of assessing PON1 gene expression or activity are either labor intensive, cumbersome for testing numerous samples, expensive and/or non-homogenous in nature.

The compound 7-diethyl-phospho-6, 8-difluoro-4-methylumbelliferyl (DEPFMU) is a substrate for PON1 with 100-fold greater sensitivity for detecting PON1 activity as compared to paraoxon [34]. PON1 catalyzes removal of the diethyl phosphate group from DEPFMU using its phosphotriesterase activity. Upon hydrolysis, the non-fluorescent DEPFMU molecule becomes 6, 8-difluoro-4-methylumbelliferyl, a highly fluorescent molecule. DEPFMU was also shown to be highly selective for PON1 in serum and, most importantly, in the presence of CHO cell lysate [34]. CHO cells over-expressing transfected PON1 gene had a significantly higher signal than non-transfected control cell lysates. A major advantage of DEPFMU over prior substrates is the low hydrolysis by cellular phosphatases and its high specificity for organophosphatases such as paraoxonase.

The goal of this research was to develop a high throughput cell-based assay to measure endogenous PON1 activity in order to rapidly screen for activators of PON1 gene expression or catalytic activity/stability. In order to detect cell-associated endogenous PON1 activity, a very sensitive and specific substrate was required. However, paraoxon is a poor substrate for PON1 in terms of turn-over rate and phenyl acetate lacks specificity for PON1 in a complex environment of cells and media. In addition, the use of absorbance in the presence of whole cells could be problematic, especially for phenyl acetate in which the product is monitored at 270 nm, the wavelength where proteins also absorb. Thus, an alternative substrate was required that was more sensitive than traditional PON1 substrates and selective for PON1 even in the presence of cells. Therefore, we employed DEPFMU as a paraoxonase specific substrate to detect endogenous PON1 activity in whole cells.

Materials and Methods

Materials

All common reagents such as Tris-HCl, CaCl₂, 2-hydroxyquinoline (2HQ) and dimethyl sulfoxide (DMSO) were reagent grade quality obtained from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO). The substrate diethylphospho 6, 8 difluoro-4-methylumbelliferone (DEPFMU) was purchased from the Invitrogen (Carlsbad, CA)

custom organic synthesis service. Black 384-well clear bottom Cell-Bind plates (catalog #3683) were purchased from Corning Incorporated (Corning, NY). Quercetin, aspirin, simvastatin and 1-chloro, 2-4 dinitrobenzene (CDNB) were obtained from Sigma-Aldrich. Human IL-1 β was purchased from Peprotech (Rocky Hill, NJ). Cell culture media components were obtained from Thermo Fisher Scientific. Huh7 cells were a gift from Masahiko Negishi at NIEHS (RTP, NC). Fresh normal rat hepatocytes were purchased from Cellzdirect/Invitrogen (RTP, NC).

Methods

PON1 Cell-Based Assay—The Huh7 cell line was cultured in at 37°C under humidified atmosphere and 5% carbon dioxide in culture media. Culture media consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. Cells were harvested for assay with trypsin/EDTA solution (Versene, Fisher). Cell density was determined by automated cell counter (Vi-CELL). For counting, equal volumes of cells and trypan blue solution (1%) were mixed and viable cells counted. The required numbers of cells were centrifuged, the media removed and the cell pellet was washed twice with phosphate buffered saline (PBS). Cells were resuspended in assay media which was DMEM/F12 (1:1) phenol red free medium (Invitrogen or Fisher) containing 0.1% FCS. The cells were then plated in black 384-well clear bottom assay plates (Corning Cell-Bind surface).

The assay was developed with a final volume of 70 μ l and in 384-well plate format to match the intended screening format. All stock solutions of compounds were made in 100% anhydrous DMSO. The typical cell based assay was performed by plating 8,000 or 10,000 Huh7 cells per well in 30 μ L of assay media (or 40 μ L when no test substance was used). Cells were allowed to adhere for 4 hrs after plating, then 10 μ l of test substances (cytokines or compounds) or solvent controls were added and then incubated for another 18 hrs. The total incubation time at 37°C following plating the cells was 22 or 24 hr, with 22 hr used as the final protocol. Thus, during the 18 hr treatment period, the final volume was 40 μ l and 0.1% DMSO when compounds were tested. After this overnight incubation, 10 μ l of 70 mM EDTA (10 mM final concentration) or 700 μ M 2HQ (100 μ M final concentration) in assay buffer (50 mM Tris pH 8.0 and 1 mM CaCl₂) was added to one set of control wells to determine background activity and 10 μ L of assay buffer added to the other wells. A 20 mM DEPFMU stock solution was made in 100% DMSO, aliquoted and stored at -20°C. DEPFMU stock solution was diluted into substrate dilution buffer (50 mM Tris pH 8.0, 1 mM CaCl₂, 1.75 M NaCl) immediately before use. 20 μ l of this DEPFMU solution was added to each well making a final assay volume of 70 μ L per well. The final substrate concentration was 40 or 80 μ M. The final concentration of NaCl was 500 mM (not accounting for salts from the assay media). Immediately after initiation of the reaction with DEPFMU, the plate was read in kinetic mode in a BMG PheraSTAR plate reader for 10 minutes. The software was programmed to read each well once per minute with 10 flashes per well, gain set at 300 and focal height of 4.3 mm with auto adjustment selected. Fluorescence data was analyzed using the plate reader software which used linear regression to generate velocities measured in RFU/min. Each data point is the average of three replicates and error bars indicating standard deviation are included on all graphs, except for the western blot quantitation. Data presented are representative of at least three independent experiments unless otherwise indicated.

Background activity was determined by addition of EDTA or 2HQ just prior to DEPFMU addition. Some lots of DEPFMU had higher initial background velocities than other lots. Diluting the DEPFMU in the substrate dilution buffer and waiting 30 min before addition to cells lowered this higher background. For the EC₅₀ data, the difference between DMSO only (untreated control) and DMSO + EDTA treated cells (background) was used to calculate

specific PON1 activity, with background subtracted from every well. The untreated wells were equated to 100% activity.

For the experiments with fresh rat hepatocytes, the cell suspension was pelleted and cells were brought up into assay media supplemented with 1x insulin/transferrin/selenium solution (Sigma-Aldrich) and plated as outlined. PON1 activity was measured 5-6 hrs after plating as per standard protocol outlined above.

IC₅₀/EC₅₀ value determinations—IC₅₀ was defined as the concentration of inhibitor that generates a 50% reduction in assay signal. EC₅₀ was defined as the concentration of compound that generates half-maximal increase in specific assay signal over untreated controls. Serial dilutions of compounds for EC₅₀ determinations were done in 100% DMSO then subsequently diluted into assay media for addition to the cells 4 hrs after plating. For the 2HQ IC₅₀ determinations, serial dilutions of 2HQ were performed in 100% DMSO then diluted into assay buffer and added to cells immediately before addition of DEPFMU. IC₅₀/EC₅₀ values and Hill slopes were calculated using a four parameter dose response (variable slope) equation. All statistical analyses were performed with GraphPad Prism software.

Western Blot Analysis—Huh7 cells were seeded into 6-well plates in DMEM/F12 phenol-free media supplemented with 1% or 0.1% FCS. After 4 hours, the cells were treated with 100 μ M aspirin, 30 μ M quercetin, 25 μ M simvastatin or 0.05% DMSO alone for 18 hours. The cells were harvested in RIPA buffer supplemented with protease and phosphatase inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA) and protein concentrations were measured with the bicinchoninic acid assay (Pierce, Rockford, IL). 55 μ g of whole cell lysates were separated by 10% or 12% NuPage Novex Bis-Tris gels (Invitrogen, Carlsbad, CA) under denaturing and reducing conditions and transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen) with the XCell II Blot module (Invitrogen). The membranes were blocked with Tris buffered saline plus 0.1% Tween-20 (TBS-T) and 0.2% I-Block (Tropix, Bedford, MA) and incubated with 4 μ g/mL PON1 monoclonal antibody (Abcam, Cambridge, MA) or β -actin monoclonal antibody (Sigma) overnight at 4°C. The following day, the membranes were washed in TBS-T and probed with HRP-conjugated goat anti-mouse secondary antibody (Pierce) for one hour at room temperature. After a final set of washes with TBS-T, HRP-conjugated SuperSignal West Pico chemiluminescent substrate (Pierce) was added to the membranes and immunoreactivity was visualized with the Kodak Image Station 4000R and analyzed with the Kodak MI Image Analysis software (Carestream Health, New Haven, CT).

Results and Discussion

Toward the goal of developing a homogeneous cell based assay for PON1 expression and activity, we determined whether hydrolysis of the substrate DEPFMU could be detected using whole cells expressing only endogenous PON1. The human hepatoma cell line Huh7 was used as a model cell that expresses PON1. As per our standard protocol, Huh7 cells were seeded into 384-well clear bottom black plates at 8,000 cells/well in 40 μ L of phenol red free DMEM/F12 (1:1) culture media and incubated for 22 hrs at 37°C. After this overnight incubation, 10 μ L of EDTA in assay buffer (50mM Tris pH 8.0 and 1 mM CaCl₂) was added to control wells. This control was used to determine background activity since EDTA is known to completely inhibit PON1 activity. The final EDTA concentration was 10 mM after DEPFMU addition. The same volume of assay buffer without EDTA was added to the rest of the wells. Subsequently, 20 μ L of DEPFMU in substrate dilution buffer (50 mM Tris pH 8.0, 1 mM CaCl₂, 3.5 M NaCl) was added making a final assay volume of 70 μ L per well. The change in fluorescence was monitored with a fluorescence plate reader. The time course of this cell based assay was examined for a 60 minute time period by measuring fluorescence every minute using 40 μ M DEPFMU (Fig. 1A). A linear increase in relative fluorescence units (RFU) was observed

for up to approximately 30 minutes after addition of substrate to the cells. In the presence of EDTA, the rate of increase slowed significantly, but a detectable rate of increase in fluorescence was still observed. Slightly higher rates were noted beyond 30 minutes in the absence of EDTA. This increase in rate was probably due to an increase in plate temperature caused by the plate remaining in the reader during the time course. Thus, it may be feasible to further enhance the sensitivity of PON1 detection by raising the assay temperature above room temperature. However, this higher temperature may introduce temperature gradients in the assay plate that would cause increased well-to-well variability across the plate – e.g. edge effects. In order to measure initial rates and avoid potentially increased variability at longer times, we chose to measure the change in fluorescence during the first 10 minutes following addition of DEPFMU to the cells. A K_m of 100 μM was reported for DEPFMU in a PON1 biochemical assay [34]. DEPFMU concentrations of 0 – 80 μM were tested in the assay with and without EDTA to determine the effect of DEPFMU concentration on the specific velocity measured in RFU/min (Fig. 1B). An increase in velocity was observed with increasing DEPFMU concentration. In all subsequent experiments, we used 40 or 80 μM substrate concentration, with 40 μM DEPFMU used for our standard assay conditions.

Although DEPFMU has been reported to be specific for PON1 in lysates, this data was obtained with CHO cells over-expressing PON1. In contrast, we used a different cell line, Huh7, and needed to measure endogenous PON1 where the signal was much closer to background. Thus, we sought to verify that the cell-dependent velocities that were being measured were indeed due to PON1 activity and not other cellular esterases. We examined the specificity of the signal generated using DEPFMU by comparing the reaction velocities with and without cells (media alone $\pm$ 0.1% FCS) and using the known PON1 inhibitor 2-hydroxyquinoline (2HQ) to specifically inhibit PON1 activity (Fig. 2). The 2HQ (100 μM) and EDTA (10 mM) were added to the cells immediately prior to addition of substrate followed by detection in the plate reader. The EDTA and 2HQ treated cells generated velocities that were indistinguishable from that of media $\pm$ FCS without any cells present. This complete inhibition of cell-dependent signal by EDTA and 2HQ suggested that the activity was divalent cation dependent and most importantly, suggested that all the cell dependent activity was due to the PON1 enzyme. Complete inhibition by the 2HQ inhibitor would not be expected if multiple esterases were responsible for the observed activity. It should be noted that a significant velocity (typically 20 - 30 RFU/min at 40 μM DEPFMU) was observed in the absence of cells. This background was the same regardless of the presence of 0.1% FCS indicating that this background velocity is not due to bovine PON1 in the serum. The serum used in this assay was heat inactivated which apparently inactivated all measurable serum PON1 activity at the tested concentration. The significant velocity observed with media alone suggested that DEPFMU undergoes auto-hydrolysis in aqueous solution and therefore this background needs to be considered in determining specific activity due to PON1. This data also established that EDTA could be used to establish valid background signal for the assay. The background velocity for every experiment was determined with either EDTA or 2HQ so that the fraction of the total velocity of the reaction that can be ascribed to PON1 can be identified. This background was also subtracted from total velocities to obtain specific PON1 activity values used in calculating percent specific PON1 activity relative to a control.

In order to further validate that the observed activity was due to PON1, we performed IC_{50} determinations with the addition of varying concentrations of 2HQ to cells just prior to substrate addition (Fig. 3). The assay was performed at a constant 0.1% DMSO at all concentrations. The average IC_{50} value and standard deviation obtained for 2HQ using this cell-based assay was 5.4 ± 3.6 μM which is close to the published K_i value of 3 μM for 2HQ with purified PON1 [35]. The average Hill slope value for these determinations was 1.4. This value is close to the theoretical value of 1.0 that is expected when all enzyme activity observed in an assay is due to a single enzyme. Furthermore, the complete inhibition of assay signal to background level

observed in the 2HQ IC₅₀ determinations indicated that all the specific signal was sensitive to 2HQ inhibition. If the observed signal in this assay was due to more than one enzyme, the inhibition by 2HQ would be expected to result in a broad slope of less than 1.0 and/or incomplete inhibition. Taken together, this data indicated that this cell-based PON1 assay is measuring PON1, and only PON1, enzymatic activity.

Optimization and characterization experiments were performed with the assay to determine optimal parameters for testing PON1 modulators. It has been reported that high concentrations (1 to 2 M) of sodium chloride enhances the PON1 catalytic activity detected in serum samples [36,37]. In order to increase the specific signal in the assay, the assay was optimized with regard to NaCl concentration present during DEPFMU hydrolysis (Fig. 4A). NaCl was added to cells at the same time as addition of DEPFMU, followed immediately by activity measurement. Background signal at each concentration of NaCl was determined by addition of 100 μ M 2HQ. Enhanced reaction velocities were observed with the addition of 250 and 500 mM NaCl (final concentrations), with 500 mM resulting in approximately a two to three-fold increase in specific activity and no increase in the background signal. The indicated concentrations are based on the added NaCl, not including contribution from media components. Therefore, we chose to use include NaCl in the buffer used to dilute DEPFMU so as to result in 500 mM NaCl when DEPFMU is added to the cells. Except for this NaCl titration experiment, all the experiments presented in this report were done at this NaCl concentration. Of note, this enhancement of enzyme activity is also consistent with the literature reporting enhanced activity of serum PON1 at high NaCl conditions.

The tolerance for FCS in the assay was determined as well. FCS used in growth media contains bovine PON1 which could add activity to the total signal and obscure the activity derived from the cells. Therefore, we tested media containing 0 - 10% serum (Fig. 4B). This media was tested with and without cells to determine the serum tolerance of the assay. In the absence of cells (media alone) there was no significant difference between any of the FCS concentrations and serum-free media. Thus, FCS concentrations of 10% or less contribute no significant PON1 activity to the assay as used for this protocol where the FCS undergoes incubation at 37°C overnight. This lack of activity observed in FCS is probably due to heat inactivation of the FCS before use. This heating appears to have inactivated most of the serum PON1 and the 22 hr incubation in the incubator during the assay may also contribute to the loss of bovine PON1 activity. In the presence of cells, the signal in the presence of 0, 0.01, and 0.1% FCS concentrations were similar, but 1% and 10% FCS resulted in a decrease in activity. The 10% FCS condition resulted in 50% reduction in signal. This loss of activity may be due to quenching the fluorescent signal by the FCS or overgrowth of the cells in the well. If cell crowding is causing the decrease in signal, then plating fewer cells, such as 4,000 cells/well, may allow use of higher FCS concentrations without a decrease in signal. We chose to use 0.1% FCS in our standard protocol to avoid a decrease in signal and minimize test compound sequestering by high protein concentrations in the assay, while providing some FCS for maintenance of good cell viability.

In order to determine optimal number of Huh7 cells for maximal PON1 activity, we tested a range of cell densities from 2,000 to 14,000 cells/well in the assay (Figure 4C). EDTA treatment was used to establish background at 10,000 and 14,000 cells/well. The PON1 activity increased in a linear fashion proportional to the number of Huh7 cells up to 10,000 cells/well and decreased at higher cell densities. The sensitivity of the assay method allowed detection of PON1 activity using as few as 2,000 cells/well - the lowest number of cells tested. Cell concentrations above 10,000 cells/well resulted in a decrease in signal most likely due to over crowding of the well that may lead to reduced cell viability. Thus, 8,000 and 10,000 cells per well were used in the assay, with 8,000 cells/well adopted for the final protocol.

One potentially significant advantage of this cell-based PON1 activity assay is that it could be readily applied to any PON1 expressing cells, including normal hepatocytes. To obtain proof-of-principle data that PON1 can be detected on normal hepatocytes, different numbers of rat hepatocytes were added to wells and DEPFMU added 5 - 6 hr later (Fig. 4C). For 5,000 and 10,000 cells per well, the reaction velocities were comparable to that observed using Huh7 cells. Above 10,000 cells/well, the velocities leveled off (15,000 cells/well) and declined at the highest concentration of cells tested (20,000 cells/well). This effect is likely due to over-crowding in the well, similar to the effect observed for Huh7 cells. These velocities were reduced to background levels in the presence of 2HQ indicating that PON1 activity was being measured. Thus, this assay is amenable for measuring endogenous cell-derived PON1 activity in normal hepatocytes. Therefore, activity of PON1 modulators in normal hepatocytes can be assessed to confirm observations made using cell lines.

Since test compounds are frequently dissolved in the solvent DMSO, the tolerance of the assay for DMSO was determined (Fig. 4D). Thus, 4 hr after plating the cells, DMSO at various concentrations was added to the cells followed by 18 hours in the incubator. There was no inhibition of PON1 activity at 0.125% DMSO, but higher concentrations showed a steady decline in PON1 activity. Thus, a final concentration of 0.1% DMSO was added to the cells 4 hr after plating when testing compounds dissolved in DMSO. The DEPFMU was stored as a stock solution in 100% DMSO, so an addition 0.1% DMSO was present at the read time. Addition of DMSO immediately before DEPFMU addition also showed significant inhibition by <1% DMSO (data not shown). This data is consistent with reports that PON1 is very sensitive to inhibition by organic solvents, including DMSO [30].

The effective concentration (EC_{50}) values for some known activators of PON1 gene expression were determined using Huh7 cells to aide in validating this assay and demonstrate its potential for identifying substances that up-regulate PON1 expression. Aspirin, quercetin, and simvastatin have all been reported to enhance PON1 gene expression by increasing transcription at the PON1 promoter [19,20,22,26]. We tested these known activators for their effect on PON1 activity with our cell-based PON1 assay. Cells were treated with compound for 18 hr (cells treated 4 hr after plating), with a final concentration of 0.1% DMSO at every concentration. The difference between untreated (DMSO only) and DMSO + EDTA treated cells was used to calculate specific PON1 activity, with background subtracted from every well and the untreated wells equated to 100% activity.

We tested the effect of aspirin on the PON1 activity expressed by Huh7 cells (Fig. 5A). Cells were treated with 3.9 to 500 μ M aspirin resulting in an approximately 200% increase in overall PON1 activity at the highest concentrations of aspirin. The average calculated EC_{50} for aspirin was 19 μ M. Thus, aspirin enhanced PON1 activity to a comparable maximum as reported in the literature which was approximately 2-fold using HepG2 cells and p-nitrophenol acetate to measure cell-associated PON1 activity in a non-homogeneous assay format [26]. However, an apparent difference in potency was observed from the literature report where only a 1.2 fold induction was observed with HepG2 cells at 100 μ M aspirin where only secreted PON1, not cell-associated, activity was measured. In contrast, we observed a 1.8-fold near-maximal increase at just 62 μ M aspirin. This difference may be due to the different responses of the Huh7 and HepG2 cells lines, or it may be that secreted PON1 does not reflect the total PON1 made. In the literature report that described the effect of aspirin on PON1 expression, the authors noted a lack of a dose response using aspirin. In contrast, we observed a clear dose response under our conditions.

An EC_{50} determination was performed using quercetin starting at a high concentration of 200 μ M (Fig. 5B). Similar to the aspirin data, a two-fold increase in overall PON1 activity was observed at the highest concentrations of quercetin. Quercetin was reported to enhance cell-

associated PON1 activity by 1.5-fold using phenyl acetate as the substrate in a non-homogeneous assay format with the Huh7 cell line [22]. The average EC_{50} value obtained for quercetin in our assay was 6.0 μ M. This value is consistent with an apparent EC_{50} of approximately 5 μ M extrapolated from a PON1 promoter luciferase assay [22]. In this same article, PON1 mRNA levels were reported to increase with quercetin treatment by approximately two-fold using Northern blot analysis. Thus, the 2-fold activation of PON1 activity observed in this assay in response to quercetin is consistent with what has been reported using standard assays.

Simvastatin was tested in this PON1 cell-based assay to further validate assay performance (Fig. 5C). Huh7 cells were treated with various concentrations of simvastatin starting at a high concentration of 100 μ M. Simvastatin treatment resulted in a maximal 2-fold increase in total PON1 activity at the highest concentrations. The average calculated EC_{50} value for simvastatin was 6.8 μ M. These results are consistent with published data wherein a maximal 2.5 or 6-fold enhancement of PON1 promoter activity was observed in luciferase assays using HepG2 cells treated with 28 or 50 μ M simvastatin [19,20]. Deakin et. al. reported a titration of simvastatin in a PON1 promoter luciferase assay resulting in an approximate EC_{50} of 7 μ M using HepG2 cells [19]. Thus our EC_{50} data was consistent with a totally different assay format that measures only transcription from the PON1 promoter.

For high throughput screening, good assay variability is an important assay quality. As a preliminary assessment of variability for this assay, we used the lowest and highest concentration data points from the IC_{50} data as representative of the negative (DMSO only) and positive (+activator) controls, respectively. These are the controls that would be used on a screening plate to establish an assay window. Aggregating all the IC_{50} data in this way resulted in average %CVs of 5.4% for the top of the window (+activator) and 3.5% for the bottom of the assay window (representing DMSO control). A common method to assess variability of an assay for screening is to calculate the Z'-factor from large scale automated variability studies. Z'-factor values in the range of 0.5 – 1.0 indicate that an assay is robust for screening [38]. Using the same data set as for the %CV calculations, a preliminary Z'-factor of 0.5 was calculated indicating a robust assay that has potential as a screening assay.

This PON1 assay uses whole cells that could release a variety of esterases upon cell death triggered by toxic test compounds. DEPFMU is reported to be selectively hydrolyzed by PON1 even in the presence of whole cell lysate derived from CHO cells [34]. However, the assay described herein uses a different cell line and is detecting endogenous PON1 instead of over-expressed PON1. Therefore, the toxic compound 1-chloro, 2-4 dinitrobenzene (CDNB) was used to determine the effect of a highly toxic compound on the assay signal (Fig. 5D). Cells were treated with various concentrations of CDNB. High doses would cause acute toxicity whereas low doses might cause delayed toxicity. A CDNB concentration dependent decrease in assay signal was observed, with 10 μ M CDNB resulting in complete inhibition of specific assay signal. No concentration of CDNB resulted in a significantly higher signal than the DMSO treated control. Therefore, most acutely toxic compounds tested with this assay will probably result in inhibition of signal rather than appear as false activators of PON1 expression.

An increase in PON1 activity was observed for cells treated with aspirin, quercetin and simvastatin. In order to confirm that the observed increase in DEPFMU hydrolysis correlated with an increase in PON1 protein level, western blot analysis was performed on cells treated with these known activators of PON1 gene expression (Fig. 6). Huh7 cells were treated with DMSO, aspirin (100 μ M), quercetin (30 μ M) or simvastatin (25 μ M) in a similar manner as in the activity assay except 6-well plates were used and cell lysates were made from the cells at the end of the treatment period. The cell lysates were analyzed by western blots with anti-PON1 antibody followed by anti-actin antibody for normalization. Normalized PON1 expression

values indicated a 1.7 to 2.3-fold increase in PON1 protein present on the cells after treatment with aspirin, quercetin and simvastatin compared to DMSO-treated control cells. Thus, the western blot data correlated with the cell-based homogenous assay in a quantitative fashion for these compounds.

There are some potential false positive artifacts that may arise in using this assay for screening a diverse collection of compounds. It is possible that a mildly toxic compound that causes cell death near the time of assay measurement may cause a false positive increase in DEPFMU hydrolysis. Another type of false positive might be a compound that activates the expression of a different esterase that can hydrolyze DEPFMU. Since this is a fluorescence-based assay, highly fluorescent test compounds may interfere with an accurate determination of activity, though the kinetic nature of the assay should prevent weakly fluorescent compounds from appearing as activators. It should also be noted that the mechanism of any initial hit is unknown since an active compound could directly or indirectly activate transcription of the PON1 gene or just enhance PON1 catalytic activity or enzyme stability. As with most homogeneous screening assays, the activity of any hits from a screen would need to be confirmed using an independent method such as western blot and/or luciferase assay. In addition, the mechanism of action would need to be determined by follow-up studies.

There are several lines of evidence that indicate that this assay measures endogenous PON1 activity. The 2HQ inhibition characteristics and the correlative data using known activators of PON1 transcription demonstrated a qualitative and quantitative reflection of PON1 activity by the assay. IL-1 β treatment of cells down-regulated the observed PON1 activity in the assay (data not shown). This result is consistent with literature reports and our own quantitative RT-PCR experiments (data not shown) that this cytokine suppresses PON1 transcription. The enhancement of activity observed with higher NaCl concentration is also consistent with literature reports for PON1. In sum, the data supports that this cell-based assay measures endogenous cell-derived PON1 activity. Thus, this activity can be a valid reflection of PON1 gene expression, though other mechanisms of activation are possible.

As a screening tool for compounds or substances that activate PON1 expression and/or activity, this assay has significant advantages over other methods. In contrast to tube based methods (where cells are incubated with substrate then cells removed by centrifugation) or multi-step laborious western blots, the described assay is significantly simpler to perform. In addition, these other methods of assessing endogenous cell-associated PON1 activity are so cumbersome that they are rarely used for generating dose response data whereas quantitative potencies, e.g. 10 point EC₅₀ data, can be rapidly generated using the described assay. The assay is homogeneous in nature and has been developed from the start in 384-well plates, characteristics that make this assay amenable for high throughput screening of compound collections for activators of PON1 expression or activity. An added benefit for this assay in screening mode is that acutely toxic compounds would likely inhibit signal and not register as false positive enhancers of PON1 activity. Since an increase in PON1 activity would be sought in a screen, it is expected that fewer false positives would arise compared to inhibition-type assays. Another high throughput format that could be used to detect activators of PON1 gene expression is the luciferase assay with the PON1 promoter. However, in addition to reporting enhanced PON1 gene transcription resulting in higher protein levels, our assay can also detect modulators of PON1 catalytic activity or stability. Neither of these mechanisms is detectable with the luciferase assay. Also, the described assay potentially avoids artifacts that can and do arise in the use of luciferase reporter assays. In addition, our assay is less expensive, simpler and higher throughput than luciferase assays that rely on transient transfections. Moreover, the suppression of PON1 activity observed when Huh7 cells were treated with IL-1 β indicates that the assay may be used to study the effects of cytokines on PON1 expression. This assay could also be used to screen natural product extracts for ability to enhance or protect PON1 activity in cells.

In addition, this novel assay could potentially be used for any cell expressing sufficient PON1. We have provided proof-of-principle data demonstrating a detectable assay signal using normal rat hepatocytes. Thus, this assay may have the unique ability to assess the effects of compounds or biomolecules on normal hepatocytes in a rapid high throughput manner. Activity in normal hepatocytes may better reflect *in vivo* efficacy to enhance PON1 expression.

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References

1. van Himbergen TM, van Tits LJ, Roest M, Stalenhoef AF. The story of PON1: how an organophosphate-hydrolysing enzyme is becoming a player in cardiovascular medicine. *Neth J Med* 2006;64:34–38. [PubMed: 16517986]
2. Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. *Nat Genet* 1996;14:334–336. [PubMed: 8896566]
3. Holvoet P. Oxidized LDL and coronary heart disease. *Acta Cardiol* 2004;59:479–484. [PubMed: 15529550]
4. Durrington PN, Mackness B, Mackness MI. Paraoxonase and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2001;21:473–480. [PubMed: 11304460]
5. Rosenblat M, Gaidukov L, Khersonsky O, Vaya J, Oren R, Tawfik DS, Aviram M. The catalytic histidine dyad of high density lipoprotein-associated serum paraoxonase-1 (PON1) is essential for PON1-mediated inhibition of low density lipoprotein oxidation and stimulation of macrophage cholesterol efflux. *J Biol Chem* 2006;281:7657–7665. [PubMed: 16407304]
6. Aviram M, Hardak E, Vaya J, Mahmood S, Milo S, Hoffman A, Billicke S, Draganov D, Rosenblat M. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation* 2000;101:2510–2517. [PubMed: 10831526]
7. Florentin M, Liberopoulos EN, Wierzbicki AS, Mikhailidis DP. Multiple actions of high-density lipoprotein. *Curr Opin Cardiol* 2008;23:370–378. [PubMed: 18520722]
8. Mackness MI, Mackness B, Durrington PN, Connelly PW, Hegele RA. Paraoxonase: biochemistry, genetics and relationship to plasma lipoproteins. *Curr Opin Lipidol* 1996;7:69–76. [PubMed: 8743898]
9. Deakin S, Leviev I, Gomaschi M, Calabresi L, Franceschini G, James RW. Enzymatically active paraoxonase-1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism. *J Biol Chem* 2002;277:4301–4308. [PubMed: 11726658]
10. Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, La Du BN. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J Clin Invest* 1998;101:1581–1590. [PubMed: 9541487]
11. Harel M, Aharoni A, Gaidukov L, Brumshtein B, Khersonsky O, Meged R, Dvir H, Ravelli RB, McCarthy A, Tokar L, Silman I, Sussman JL, Tawfik DS. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat Struct Mol Biol* 2004;11:412–419. [PubMed: 15098021]
12. Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* 1998;394:284–287. [PubMed: 9685159]
13. Mackness B, Quarck R, Verreth W, Mackness M, Holvoet P. Human paraoxonase-1 overexpression inhibits atherosclerosis in a mouse model of metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2006;26:1545–1550. [PubMed: 16627808]
14. Oda MN, Bielicki JK, Ho TT, Berger T, Rubin EM, Forte TM. Paraoxonase 1 overexpression in mice and its effect on high-density lipoproteins. *Biochem Biophys Res Commun* 2002;290:921–927. [PubMed: 11798161]

15. She ZG, Zheng W, Wei YS, Chen HZ, Wang AB, Li HL, Liu G, Zhang R, Liu JJ, Stallcup WB, Zhou Z, Liu DP, Liang CC. Human paraoxonase gene cluster transgenic overexpression represses atherogenesis and promotes atherosclerotic plaque stability in ApoE-null mice. *Circ Res* 2009;104:1160–1168. [PubMed: 19359600]
16. Tward A, Xia YR, Wang XP, Shi YS, Park C, Castellani LW, Lusis AJ, Shih DM. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation* 2002;106:484–490. [PubMed: 12135950]
17. Bhattacharyya T, Nicholls SJ, Topol EJ, Zhang R, Yang X, Schmitt D, Fu X, Shao M, Brennan DM, Ellis SG, Brennan ML, Allayee H, Lusis AJ, Hazen SL. Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. *JAMA* 2008;299:1265–1276. [PubMed: 18349088]
18. Arii K, Suehiro T, Ota K, Ikeda Y, Kumon Y, Osaki F, Inoue M, Inada S, Ogami N, Takata H, Hashimoto K, Terada Y. Pitavastatin induces PON1 expression through p44/42 mitogen-activated protein kinase signaling cascade in Huh7 cells. *Atherosclerosis* 2009;202:439–445. [PubMed: 18572174]
19. Deakin S, Leviev I, Guernier S, James RW. Simvastatin modulates expression of the PON1 gene and increases serum paraoxonase: a role for sterol regulatory element-binding protein-2. *Arterioscler Thromb Vasc Biol* 2003;23:2083–2089. [PubMed: 14500290]
20. Ota K, Suehiro T, Arii K, Ikeda Y, Kumon Y, Osaki F, Hashimoto K. Effect of pitavastatin on transactivation of human serum paraoxonase 1 gene. *Metabolism* 2005;54:142–150. [PubMed: 15690306]
21. Muacevic-Katanec D, Bradamante V, Poljicanin T, Reiner Z, Babic Z, Simeon-Rudolf V, Katanec D. Clinical study on the effect of simvastatin on paraoxonase activity. *Arzneimittelforschung* 2007;57:647–653. [PubMed: 18074758]
22. Gouedard C, Barouki R, Morel Y. Dietary polyphenols increase paraoxonase 1 gene expression by an aryl hydrocarbon receptor-dependent mechanism. *Mol Cell Biol* 2004;24:5209–5222. [PubMed: 15169886]
23. Gouedard C, Barouki R, Morel Y. Induction of the paraoxonase-1 gene expression by resveratrol. *Arterioscler Thromb Vasc Biol* 2004;24:2378–2383. [PubMed: 15458977]
24. Gong M, Garige M, Varatharajulu R, Marmillot P, Gottipatti C, Leckey LC, Lakshman RM. Quercetin up-regulates paraoxonase 1 gene expression with concomitant protection against LDL oxidation. *Biochem Biophys Res Commun* 2009;379:1001–1004. [PubMed: 19141295]
25. Curtin BF, Seetharam KI, Dhoieam P, Gordon RK, Doctor BP, Nambiar MP. Resveratrol induces catalytic bioscavenger paraoxonase 1 expression and protects against chemical warfare nerve agent toxicity in human cell lines. *J Cell Biochem* 2008;103:1524–1535. [PubMed: 17879943]
26. Jaichander P, Selvarajan K, Garelnabi M, Parthasarathy S. Induction of paraoxonase 1 and apolipoprotein A-I gene expression by aspirin. *J Lipid Res* 2008;49:2142–2148. [PubMed: 18519978]
27. Kumon Y, Nakauchi Y, Suehiro T, Shiinoki T, Tanimoto N, Inoue M, Nakamura T, Hashimoto K, Sipe JD. Proinflammatory cytokines but not acute phase serum amyloid A or C-reactive protein, downregulate paraoxonase 1 (PON1) expression by HepG2 cells. *Amyloid* 2002;9:160–164. [PubMed: 12408678]
28. Kumon Y, Suehiro T, Ikeda Y, Hashimoto K. Human paraoxonase-1 gene expression by HepG2 cells is downregulated by interleukin-1beta and tumor necrosis factor-alpha, but is upregulated by interleukin-6. *Life Sci* 2003;73:2807–2815. [PubMed: 14511766]
29. Van Lenten BJ, Wagner AC, Navab M, Fogelman AM. Oxidized phospholipids induce changes in hepatic paraoxonase and ApoJ but not monocyte chemoattractant protein-1 via interleukin-6. *J Biol Chem* 2001;276:1923–1929. [PubMed: 11034996]
30. Khersonsky O, Tawfik DS. Structure-reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase. *Biochemistry* 2005;44:6371–6382. [PubMed: 15835926]
31. Khersonsky O, Tawfik DS. Chromogenic and fluorogenic assays for the lactonase activity of serum paraoxonases. *Chembiochem* 2006;7:49–53. [PubMed: 16329153]
32. Han CY, Chiba T, Campbell JS, Fausto N, Chaisson M, Orasanu G, Plutzky J, Chait A. Reciprocal and coordinate regulation of serum amyloid A versus apolipoprotein A-I and paraoxonase-1 by

- inflammation in murine hepatocytes. *Arterioscler Thromb Vasc Biol* 2006;26:1806–1813. [PubMed: 16709944]
33. Osaki F, Ikeda Y, Suehiro T, Ota K, Tsuzura S, Arai K, Kumon Y, Hashimoto K. Roles of Sp1 and protein kinase C in regulation of human serum paraoxonase 1 (PON1) gene transcription in HepG2 cells. *Atherosclerosis* 2004;176:279–287. [PubMed: 15380450]
34. Soukharev S, Hammond DJ. A fluorogenic substrate for detection of organophosphatase activity. *Anal Biochem* 2004;327:140–148. [PubMed: 15033522]
35. Aharoni A, Gaidukov L, Yagur S, Toker L, Silman I, Tawfik DS. Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proc Natl Acad Sci U S A* 2004;101:482–487. [PubMed: 14695884]
36. Beltowski J, Wojcicka G, Marciniak A. Species- and substrate-specific stimulation of human plasma paraoxonase 1 (PON1) activity by high chloride concentration. *Acta Biochim Pol* 2002;49:927–936. [PubMed: 12545199]
37. Eckerson HW, Romson J, Wyte C, La Du BN. The human serum paraoxonase polymorphism: identification of phenotypes by their response to salts. *Am J Hum Genet* 1983;35:214–227. [PubMed: 6301268]
38. Zhang J, Thomas D, Chung Y, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67–74. [PubMed: 10838414]

Fig. 1.

Time course and substrate titration. A. Cells were plated into 384-well plates and incubated for 22 hr followed by the addition of EDTA (▲) or buffer (■), followed by the addition of DEPFMU (40 μM final concentration). The fluorescence of the wells was measured once per minute. The fluorescence in RFU versus time is shown. B. Various substrate concentrations as indicated were added to the wells in the presence (▲) or absence (■) of EDTA. The velocities of the reaction in RFU/min were obtained from the first ten minutes of the reaction by reading fluorescence once per minute. Data points were performed in triplicate determination and error bars represent standard deviations. Data are representative of at least two independent experiments.

Fig. 2.

Specificity of assay signal. Assay media with and without FCS was tested for ability to hydrolyze DEPFMU in the absence of cells, as indicated. Cells in assay media (8,000/well) were incubated for 22 hr in assay media and then treated with 10 mM EDTA, 100 μ M 2HQ, or buffer (UT, untreated) followed immediately by the addition of DEPFMU in assay buffer and measurement in a plate reader for 10 min. Data points were performed in triplicate determination and error bars represent standard deviations. Data are representative of three independent experiments.

Fig. 3.

Inhibition of assay signal by 2HQ. The IC_{50} and Hill slope values for 2HQ were determined by addition of the indicated concentrations of 2HQ to cells followed immediately by the addition of DEPFMU (80 μM final) and velocity measurements. Final DMSO concentration in all wells was 0.1%. Data points were performed in triplicate determination and error bars represent standard deviations. Data are representative of three independent experiments.

Fig. 4.

PON1 cell-based assay optimization and characterization. (A) The cell-based assay was optimized with regard to NaCl concentration at the time of detection of DEPFMU hydrolysis. NaCl was added to cells at the same time as addition of DEPFMU, followed immediately by activity measurement (■). The indicated concentrations are final concentration added, not including contribution from media components. Background signal at each concentration of NaCl was determined by addition of 100 μ M 2HQ (▲). (B) The effect of various concentrations of FCS was examined by plating cells or media alone (no cells) in 0 – 10% FCS. (C) The number of cells per well was optimized by addition of the indicated number of Huh7 cells to wells (■, solid line). Background signal was established by addition of 10 mM EDTA to the wells containing 10,000 Huh7 cells (▲). In a separate experiment, freshly isolated normal rat hepatocytes (dashed lines) were added to plates at the indicated number of cells per well. After 5-6 hours at 37°C, DMSO (●) or 2HQ (◆) was added to the cells immediately followed by the addition of DEPFMU and detection of fluorescence as usual. (D) The DMSO tolerance of the assay was determined by addition of the indicated concentrations of DMSO four hours after plating the cells. The dotted line indicates background signal as established by 10 mM EDTA. Data points were performed in triplicate determination and error bars represent standard deviations. Data are representative of at least three independent experiments, except for the hepatocyte data which are representative of two independent experiments

Fig. 5.

Compound EC_{50} value determinations using Huh7 cells. The EC_{50} values were determined for aspirin (A), quercetin (B), simvastatin (C) and CDNB (D) by addition of the indicated concentrations of compound to cells four hours after cell plating followed by an 18 hr incubation and then DEPFMU hydrolysis measured per standard protocol. Serial dilutions were performed in 100% DMSO. Final DMSO concentration was 0.1% in all wells. Percent specific activity was calculated based on velocities of control wells that received only DMSO (100% activity) and background velocity, as determined by treatment with DMSO + EDTA, was subtracted from all wells. Data points were performed in triplicate determination and error bars represent standard deviations. Data are representative of three independent experiments.

Fig. 6. PON1 protein expression analysis. (A) Cells were plated and treated with 100 μ M aspirin, 30 μ M quercetin, 25 μ M simvastatin, or 0.05% DMSO for 18 hours. Whole cell protein lysates were analyzed by Western blot analysis with anti-PON1 monoclonal antibody. In this representative Western blot, top bands are PON1 protein and the bottom bands resulted from the same blot stripped and re-probed with anti- β -actin monoclonal antibody. (B) The net intensities of the bands were digitally analyzed and the ratio of PON1: β -actin bands were calculated and represented graphically. Data are representative of two independent experiments.