Mitochondrial targeting of the electrophilic lipid 15-deoxy-Δ^{12,14}-prostaglandin J₂ increases apoptotic efficacy via redox cell signalling mechanisms

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INTRODUCTION

Electrophilic compounds have attracted great interest as potential therapeutic agents in several fields, including cancer, cardiovascular disease and neurodegeneration [1–4]. Their primary mechanism of action occurs through their ability to covalently modify key proteins in redox cell signalling pathways [5–7]. Electrophiles can be derived from several sources including the diet, environment or endogenously through enzymatic or non-specific lipid peroxidation processes [4,8]. Endogenously generated electrophiles can be formed through enzymatic pathways, most notably via cyclo-oxygenase. Arachidonic acid oxidation results in the formation of a family of lipid prostaglandins, some of which are electrophilic in nature [9,10]. These electrophilic cyclo-oxygenase metabolites have been shown to possess anticancer, anti-inflammatory and cardioprotective properties [11–13].

Indeed, one of the best characterized electrophilic lipids generated from cyclo-oxygenase is 15d-PGJ₂ (15-deoxy-Δ^{12,14}-prostaglandin J₂). It is particularly interesting because of its high degree of specificity for modification of protein cysteine residues (thiols) in signalling proteins [14]. For example, 15d-PGJ₂ has been shown to increase expression of phase II detoxification enzymes and cytoprotective intracellular antioxidants such as HO-1 (haem oxygenase-1) and the enzymes responsible for GSH synthesis [14–16]. Although 15d-PGJ₂ is also known as a PPARγ (peroxisome-proliferator-activated receptor γ) agonist, the induction of HO-1 and GSH requires the presence of the electrophilic reactive centre in the lipid and is not recapitulated by other non-reactive PPARγ agonists [15]. We have termed the highly selective family of proteins that form covalent adducts with electrophilic lipids the ‘electrophile-responsive proteome’ [17]. This is a discreet proteome given that of the several hundred proteins in a cell which have potentially reactive thiols only approx. 10% form covalent protein adducts with 15d-PGJ₂.

In a therapeutic context, 15d-PGJ₂ has been proposed as both a potential anticancer and anti-inflammatory agent because of its ability to regulate redox-sensitive aspects of angiogenesis, growth arrest, expression of inflammatory cytokines and cell death through the covalent modification of proteins [1,3]. For example, it has been shown to inhibit angiogenesis through the suppression of inflammatory enzymes and cytokines; this occurs through the direct modification of key components of the NF-κB (nuclear factor κ-light-chain-enhancer of the activated B-cell) signalling pathway. Specifically, the potent anti-inflammatory effects of 15d-PGJ₂ are attributed to the covalent modification of the p50 subunit of NF-κB by 15d-PGJ₂, which results in the inhibition of its DNA-binding activity [18]. It was also found that 15d-PGJ₂ modifies two cysteine residues within the C-terminal zinc finger of ER (oestrogen receptor)-α, and this modification inhibits its
DNA-binding activity, decreases target gene expression and inhibits proliferation [19]. The cell-death effects of 15d-PGJ2 have been reported to occur through a pleiotropic mechanism involving activation of PPARγ and through interactions with mitochondrial proteins which lead to the activation of apoptosis [12,20]. It is well recognized that these redox signalling pathways may be important therapeutic targets; however, the major limitation has been the inability to selectively target the specific redox signalling pathways that regulate the beneficial effects of electrophiles.

Evidence clearly supports the fact that the protein targets of 15d-PGJ2 in the electrophile-responsive proteome are found in different compartments in the cell, including the cytosol and mitochondria, and this is consistent with reports that 15d-PGJ2 acts by pleiotropic mechanisms [18,21]. In work pioneered by Murphy and colleagues, a novel strategy for intracellular targeting of compounds to the mitochondrion has been developed. The conjugation of a delocalized, lipophilic cation to a compound of interest directs its accumulation within the mitochondrion and is sustained by the mitochondrial membrane potential [22,23]. This strategy has been employed to direct therapeutic antioxidants to the mitochondrion [24–26]. On the basis of these previous studies, suggesting that the electrophilic modification of cytosolic targets are protective, whereas modification of mitochondrial proteins may regulate apoptosis, we hypothesized that targeting an electrophile to the mitochondrion would enhance the apoptotic effects of this compound while limiting the induction of intracellular antioxidants.

To test this hypothesis, we synthesized a novel mitochondrially targeted analogue of 15d-PGJ2 (mito-15d-PGJ2) and examined its effects on apoptotic cell death and induction of intracellular antioxidants. We found that mito-15d-PGJ2 was more potent at initiating intrinsic apoptosis than 15d-PGJ2. Additionally, mito-15d-PGJ2 was less effective at up-regulating Keap1 (Kelch-like antiboxidants. We found that mito-15d-PGJ2 was more potent at its effects on apoptotic cell death and induction of intracellular prostaglandin E2 were purchased from Cayman Chemicals. EZ-depolarization when compared with 15d-PGJ2. A mitochondrially in mitochondrial bioenergetics and mitochondrial membrane ant expression (HO-1 and GSH) and caused profound defects enoyl-CoA hydratase-associated protein 1)-dependent antioxid-ation (HO-1 and GSH) and caused profound defects in mitochondrial bioenergetics and mitochondrial membrane depolarization when compared with 15d-PGJ2. A mitochondrially targeted analogue of the non-electrophilic lipid prostaglandin E2, mito-PGE2, and the targeting moiety TPMP (methyltriphenylphosphonium) did not recapitulate these effects. Taken together, these results demonstrate for the first time the feasibility of activating specific redox signalling pathways with an electrophile by selectively targeting specific compartments within the cell.

**EXPERIMENTAL**

**Materials**

All chemicals were of analytical grade and purchased from Sigma–Aldrich unless otherwise specified. 15d-PGJ2 and prostaglandin E2 were purchased from Cayman Chemicals. EZ-link 5-(biotinamido)pentylamine was purchased from Pierce Chemicals and was used for the synthesis of bi-15d-PGJ2 (biontinallyated 15d-PGJ2) as described previously [6]. TPMP bromide was used as a mitochondrial-targeting control. 2-Aminoethyl-TPMP bromide was provided by Dr B. Kalyanaraman.

**Cell culture**

MDA-MB231 human mammary adenocarcinoma cells were cultured in RPMI 1640 medium (Cellgro) containing 10% (v/v) FBS (fetal bovine serum). MCF10A-immortalized human mammary epithelial cells were cultured in MEGM (mammary epithelial cell growth medium) supplemented with MEGM

![Figure 1 Structures of lipids used in the present study](image)

**Figure 1** Structures of lipids used in the present study

Structures of 15d-PGJ2 and the mitochondrially targeted lipid derivatives mito-15d-PGJ2 and mito-PGE2. Electrophilic carbon centres are denoted with (*).

SingleQuots (Lonza). MEFs (murine embryonic fibroblasts), derived from wild-type and Nrf2 (nuclear factor-erythroid 2-related factor 2)-null mice as described previously [27], were cultured in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% (v/v) FBS and supplemented with 50 μM 2-mercaptoethanol. All experiments using MDA-MB231 cells were performed in RPMI 1640 medium with 0.5% FBS at approx. 50% confluence. Cells were cultured in six-well cluster plates except when used for bioenergetic measurements when specialized Seahorse Bioscience culture plates were used. For lipid exposures in six-well cluster plates 1 ml of medium was used (a 10 μM addition of lipid was then equivalent to approx. 160 pmols lipid per μg of protein). For lipid exposures in Seahorse Bioscience culture plates, 250 μl of medium was used (a 10 μM addition of lipid gave a similar concentration of approx. 125 pmols lipid per cell). Experiments utilizing MEFs and MCF10A cells were conducted at confluence in DMEM containing 0.5% FBS or MEGM respectively, with supplementation as described above.

**Synthesis of mito-15d-PGJ2 and mito-PGE2**

In order to target the lipids to the mitochondria, we utilized the lipophilic cation TPP+ (triphenylphosphonium) [28]. The procedure is a modification of a method reported previously [6,29] and is described in detail in the Supplementary Experimental section (available at http://www.BiochemJ.org/bj/426/ bj4260031add.htm). HPLC, UV–visible spectral analysis, and mass spectrometric confirmation of synthesized lipids are shown in Supplementary Figures S1–S3 (available at http://www. BiochemJ.org/bj/426/bj4260031add.htm). Structures for the parent compound and synthesized lipids are also shown in Figure 1.

**Assessment of cell viability**

After lipid exposure, aliquots of the medium from the treatment wells were taken, and cells were harvested by scraping in PBS lysis buffer containing 0.1% Triton X-100. After centrifuging
cell lysates (14,000 g for 10 min) to remove debris, LDH (lactate dehydrogenase) activity in the supernatant and aliquots of cell medium was measured spectrophotometrically by monitoring the oxidation of 0.3 mM NADH at 340 nm. Apoptosis and necrosis were measured, after the indicated treatment had been performed for 16 h, by flow cytometric analysis using an Annexin V FITC apoptosis detection kit (Calbiochem) using a BD Biosciences LSR II flow cytometer. Briefly, gating parameters were set using PI (propidium iodide) only, Annexin V only and no staining controls, and 10,000 events were collected for each experimental sample. Cells staining positively for both PI and Annexin V were considered late apoptotic. Cells staining positively for PI only or Annexin V only were scored as necrotic or early apoptotic respectively. Cells which were negative for both PI and Annexin V staining were scored as viable cells.

**Glutathione measurement**

The total glutathione (GSH and GSSG) level was determined in cell lysates, treated as indicated, using the recycling assay described previously [30]. Briefly, after treatment, cells were lysed in 10 μM DTPA (diethylenetriaminepenta-acetic acid) and 0.1% Triton X-100 in PBS, pH 7.4. Total glutathione in lysates was determined spectrophotometrically by monitoring the reduction of 5,5′-dithio-bis(2-nitro-benzoic acid) at 412 nm. Values were normalized to protein content assayed by the Bradford method (using a Bio-Rad Laboratories protein assay kit).

**Western blot analysis**

Cell lysate proteins were separated by SDS/PAGE (10% or 12.5% gels) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Protein amounts were quantified by the Bradford method (using a Bio-Rad Laboratories protein assay kit), and equivalent amounts of protein were loaded. Uniform protein loading was verified by Ponceau S staining of the membranes, which showed no significant differences in protein levels on the blots among samples. The membranes were blocked with 5% (w/v) dried non-fat milk/TBS-T (Tris-buffered saline with 0.05% Tween 20) solution for 1 h at room temperature (24°C), and then incubated with primary antibody overnight at 4°C or for 3 h at room temperature. The antibody dilutions used are as follows: anti-HO-1 antibody (Stressgen), 1:1000; anti-caspase 9 antibody (Cell Signaling Technology), 1:1000; anti-VDAC (voltage-dependent anion channel) antibody (Mitosciences), 1:3000; anti-β-actin antibody (Cell Signaling Technology), 1:1000; and anti-COX (cytochrome c oxidase) subunit I antibody (Mitosciences), 1:1000. After washing with TBS-T, membranes were incubated with an HRP (horseradish peroxidase)-conjugated secondary antibody (GE Healthcare). Membranes were then developed using SuperSignal West Dura chemiluminescence substrate (Pierce Chemicals) and imaged using a charged-coupled device camera imaging system (AlphaInnotech).

**Measurement of mitochondrial function**

To measure mitochondrial function in intact MDA-MB231 cells, a Seahorse Bioscience XF24 Extracellular Flux Analyzer was used [31]. The optimal seeding density of MDA-MB231 cells needed to obtain a measurable OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) was established, and both the ECAR and OCR show a proportional response with respect to the cell number (results not shown). For subsequent experiments a seeding density of 40,000 cells per well was selected to allow both potential increases and the inhibition of OCR and ECAR to be assessed. We noted that high levels of FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone] inhibited mitochondrial respiration presumably due to the loss of the ability to accumulate respiratory substrates. Accordingly, oligomycin, FCCP and antimycin A concentrations were optimized in order to elicit maximal effects in preliminary experiments (results not shown). The mitochondrial function assay employed in the present study uses sequential injections of oligomycin, FCCP and antimycin A to define a number of mitochondrial parameters such as basal OCR, ATP-linked OCR, proton leak, maximal respiratory capacity, reserve respiratory capacity and non-mitochondrial oxygen consumption. It is important to note that in order to calculate these parameters, we have assumed that the oligomycin-insensitive OCR is attributable to proton leak; however, oligomycin has been shown to hyperpolarize the mitochondrial membrane [32], and therefore the resulting OCR is probably an upper estimate of the contribution from proton leak. The mitochondrial inner membrane electrochemical potential (Δψ) was assessed using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide; Invitrogen). Briefly, cells grown in 12-well culture plates were treated as described in the Figure legends, and 7.4 μM JC-1 was added directly to the MDA-MB231 cells in culture medium and incubated for 30 min. Next, cells were washed with PBS, and the red and green fluorescence was measured using a fluorescence plate reader. Results are expressed as the ratio of red to green fluorescence.

**Protein adduct detection and mitochondrial fractionation**

Following experimental incubations, MDA-MB231 lysates were analysed by two-dimensional IEF (isoelectric focussing) SDS/PAGE. Total cell lysates (50 μg) were separated in the first dimension on a pH 3–9 gradient (IPG strips; Bio-Rad Laboratories) followed by resolution by SDS/PAGE (12.5% gels), and proteins were transferred on to nitrocellulose membranes as described previously [15]. After blocking for 1 h, the membranes were probed using an anti-TPP+ antibody (a gift from Professor M.P. Murphy, Medical Research Council Mitochondrial Biology Unit, Cambridge, U.K.) and with either an HRP-conjugated secondary antibody or with streptavidin conjugated to HRP (GE Healthcare) for detection of mito-15d-PGJ2 and 15d-PGJ2 adducts respectively. Membranes were developed as described above, and total protein was detected using Deep Purple stain (GE Healthcare). Blots were analysed using PDQuest software (Bio-Rad Laboratories). For mitochondrial fractionation, after treatment, MDA-MB231 cells were harvested by scraping into ice-cold HMIM (heart mitochondria isolation medium) buffer (20 mM Tris/HCl, pH 7.35, 300 mM sucrose and 2 mM EGTA at 4°C) containing protease inhibitor cocktail (Roche) and 1 mM PMSF as described in detail in the Supplementary Experimental section. Protein adducts were detected using reagents described above. Membranes were developed using ECL+ (enhanced chemiluminescence; GE Healthcare) and chemiluminescence was imaged using a Typhoon fluorescence detector (GE Healthcare).

**Statistical analysis**

Results are reported as means ± S.E.M. for n≥3, as indicated in the Figure legends. Statistical significance was evaluated by one-way ANOVA among the groups using GraphPad Prism 4. The minimum level of significance was set at P < 0.05. Tukey’s
multiple comparison test was used for post-hoc analysis of significance between groups.

RESULTS

Cytotoxicity of 15d-PGJ2 and mito-15d-PGJ2

It is established that lipid electrophiles at low concentrations induce phase II cytoprotective enzymes, but at higher concentrations initiate cell death [16]. For example, the parent compound in these studies, 15d-PGJ2, has been shown to induce apoptotic cell death in a number of cancer cell lines at doses ranging from 5 to 50 μM [33–35]. To assess cytotoxicity of mito-15d-PGJ2 in human mammary cells, non-tumorigenic MCF10A mammary epithelial cells and MDA-MB231 mammary adenocarcinoma cells were exposed to 3–30 μM mito-15d-PGJ2 for 16 h and then cell death was assessed by using LDH release as an indicator of late apoptotic or necrotic cell death. Treatment of both cell lines resulted in significant cell death at concentrations at or above 10 μM (Figure 2A). Additional examination of the cell-death effects of mito-15d-PGJ2 relative to 15d-PGJ2 were conducted in MDA-MB231 cells. We next determined the relative
cytotoxicity of mito-15d-PGJ₂, by treating MDA-MB231 cells with increasing concentrations (0.3–30 μM) of either mito-15d-PGJ₂ or 15d-PGJ₂ for 16 h. Cytotoxicity was assessed using FACS analysis, after staining with PI and Annexin V. Mito-15d-PGJ₂ treatment resulted in a greater decrease in viable cells (those that are not positive for either Annexin V or PI) at lower concentrations when compared with 15d-PGJ₂ (Figure 2B). Hardly any early and late apoptotic cells were detectable after exposure to 15d-PGJ₂, whereas this represented the majority of the cell population after exposure to mito-15d-PGJ₂ (Figures 2C and 2D). In contrast, 15d-PGJ₂ induced predominantly necrotic cell death at 30 μM, whereas this represented only 3–10% of the cells treated with mito-15d-PGJ₂ across the dose range (Figure 2E).

As a further confirmation that mito-15d-PGJ₂ causes apoptotic cell death, MDA-MB231 cells were treated with 10 μM 15d-PGJ₂ or mito-15d-PGJ₂ for 4 h, and then cell lysates were probed for pro-caspase 9, a protein which is cleaved upon the activation of the intrinsic (mitochondrial) apoptotic signalling. Treatment with 15d-PGJ₂ resulted in a partial loss, or activation, of pro-caspase 9, whereas mito-15d-PGJ₂ caused complete loss of this apoptotic mediator (Figure 2F). Mito-PGE₂ and TPMP were used as controls for the effect of the TPP⁺ moiety and electrophilicity and had no effect on the levels of pro-caspase 9. Staurosporine (1 μM) was used as a positive control for caspase activation. Taken together these results show that mito-15d-PGJ₂ induces cell death primarily through apoptosis, and more robustly than 15d-PGJ₂, which is consistent with the hypothesis that mitochondrial targeting then biases the mechanism of cell death towards apoptosis over necrosis.

**Figure 3 Differential induction of HO-1 and GSH by 15d-PGJ₂ and mito-15d-PGJ₂**

MDA-MB231 cells were treated with increasing concentrations (0.3–10 μM) of 15d-PGJ₂ and mito-15d-PGJ₂. (A) Cells were treated for 4 h then HO-1 protein levels were determined using Western blot analysis and the levels were quantified (lower panel). Equivalent amounts of protein were loaded for each sample and equal protein loading was confirmed after transfer on to nitrocellulose membranes by using Ponceau S staining. A representative Western blot is shown (upper panel). (B) Cells were treated for 4 h, then compound-containing medium was removed and RPMI 1640 medium containing 0.5% FBS was added for an additional 16 h. Cell lysates were then analysed for total glutathione content and values were normalized to the total protein level. Results represent means ± S.E.M., n = 3. **P < 0.01 compared with ethanol vehicle control. #P < 0.05 compared with 15d-PGJ₂.

**Nrf2 dependence of the regulation of antioxidant expression and cell death by 15d-PGJ₂**

We have reported previously that the induction of HO-1 by 15d-PGJ₂ can be inhibited by modification of mitochondrial protein thiois [16]. To test the possibility that mito-15d-PGJ₂ is functioning through a similar mechanism, cells were pretreated with mito-15d-PGJ₂ for 4 h then 15d-PGJ₂ was added to stimulate HO-1 and GSH levels. Mito-15d-PGJ₂ does not impair the 15d-PGJ₂-stimulated induction of HO-1, indicating that is not inhibiting the mitochondrial component required for HO-1 synthesis (Figure 4A). Interestingly, in the combined treatment group, mito-15d-PGJ₂ pretreatment enhances HO-1 induction by 15d-PGJ₂. These results are consistent with previous studies showing that induction of HO-1 occurs by a number of mechanisms, some of which appear to be independent of Keap1/Nrf2 [38]. In contrast, the induction of GSH appears to require Nrf2 and, consistent with this finding, pretreatment with mito-15d-PGJ₂ does not impair 15d-PGJ₂-dependent induction of GSH (Figure 4B).

It has been established that activation of the Keap1/Nrf2 system by low levels of electrophilic stress results in increased resistance
to cytotoxic levels of reactive oxygen and nitrogen species. It is then possible that the failure of mito-15d-PGJ₂ to activate the EpRE may contribute to its cytotoxicity. To test this, two different experimental strategies were employed. First, MDA-MB231 cells were co-incubated with a cell-death-inducing concentration of mito-15d-PGJ₂ (10 μM) and a concentration of 15d-PGJ₂ which increases antioxidant expression (3 μM). After a 16 h treatment, cell death was assessed by LDH release and was unchanged by co-treatment with 15d-PGJ₂ (Figure 4C). This is consistent with the early induction of the apoptotic process before significant induction of phase II cytoprotective enzymes can occur. Secondly, wild-type and Nrf2-null MEFs were treated with 3 or 10 μM 15d-PGJ₂ or mito-15d-PGJ₂ and the level of cell death was assessed. If the cytotoxicity was attenuated by activation of the Keap1/Nrf2 system, we would have expected the Nrf2-null cells to be sensitized to the electrophiles; however, we found that cytotoxicity of 15d-PGJ₂ and mito-15d-PGJ₂ was independent of Nrf2 levels in the cell (Figure 4D).

Protein modification by bt-15d-PGJ₂ and mito-15d-PGJ₂ are different

The results thus far demonstrating the different biological properties of 15d-PGJ₂ and mito-15d-PGJ₂ suggest that the proteins modified by the electrophilic lipids will also differ. To test this, MDA-MB231 cells were incubated with 10 μM bt-15d-PGJ₂ or mito-15d-PGJ₂ for 1 h, and the protein lysates separated by two-dimensional IEF SDS/PAGE followed by Western blotting and detection for protein–lipid adducts using the anti-TPP⁺ antibody or streptavidin conjugated to HRP. The patterns of protein stains for all treatments showed no significant differences (results not shown). Control cells not treated with electrophilic lipid showed no major positive reactions with the exception of one spot on the biotin blot (Figures 5A and 5B), which we ascribe to an endogenous biotin-containing carboxylase [6]. Figure 5 shows detection of approx. 63 proteins that are positive for biotin (Figure 5C) and approx. 32 proteins that are positive for TPP (Figure 5D).
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Figure 5 Visualization of bt-15d-PGJ2- and mito-15d-PGJ2-modified proteins by two-dimensional IEF SDS/PAGE

Cell lysates from MDA-MB231 cells were separated by two-dimensional IEF SDS/PAGE. Ethanol-vehicle-control-treated (1 h) cell lysates were probed using (A) streptavidin–HRP and (B) anti-TPP protein with an HRP-conjugated secondary antibody to determine background reactivity. Cells were treated (10 μM for 1 h) with (C) bt-15d-PGJ2 or (D) mito-15d-PGJ2 and protein adducts were detected using streptavidin–HRP and anti-TPP antibody respectively. Arrows indicate common spots with the same intensity that were used to select images. (E) A merged image of the bt-15d-PGJ2 (red) and mito-15d-PGJ2 (green) adduct blots. (F) Analysis of spot quantity was determined and the number of common and unique spots from bt-15d-PGJ2 and mito-15d-PGJ2 membranes is shown schematically.

The images were selected to minimize differences in potential sensitivity between the two detection techniques by selecting images with the same intensity for a common spot (indicated by an arrow on Figures 5C and 5D). The overlay of these two images was achieved using the PDQuest proteomics software (Figure 5E) and shows red spots that are predominantly reactive with bt-15d-PGJ2 and green spots that are predominantly reactive with mito-15d-PGJ2, so that the proteins that react with both lipids are yellow. In Figure 5(F), these results are summarized and indicate that ten proteins are common targets of both electrophilic lipids, with 22 targets unique to mito-15d-PGJ2 and 53 targets unique to 15d-PGJ2. It was not possible to unequivocally overlay the protein adduct maps with the protein stain to identify these targets as many of these proteins are present in the cell at low abundance [6].

15d-PGJ2 and mito-15d-PGJ2 localize to mitochondria and affect mitochondrial membrane potential

To assess the relative localization and formation of protein adducts by the lipid electrophiles, mitochondrial fractions were isolated from cells treated with bt-15d-PGJ2 or mito-15d-PGJ2. Validation of the fractionation protocol used demonstrates substantial enrichment of the mitochondrial proteins in the mitochondrial fraction compared with the cytosolic fraction (see Supplementary Figure S4 available at http://www.BiochemJ.org/bj/426/bj4260031add.htm).

Protein adduct formation was assessed in fractionated samples from bt-15d-PGJ2- and mito-15d-PGJ2-treated cells using one-dimensional SDS/PAGE. Consistent with our previous results [21], bt-15d-PGJ2 localizes to the mitochondria and forms stable protein adducts. Mito-15d-PGJ2 protein adducts are also enriched in the mitochondrial fraction (Figure 6A). Mitochondrial purity was also confirmed by the VDAC enrichment in the mitochondrial fraction. Protein adducts in each fraction were quantified and expressed as the fold increase in protein adducts in the mitochondrial fraction compared with the homogenate (Figure 6B). These results demonstrate that there is a significant increase in mito-15d-PGJ2–protein adducts in the mitochondria compared with the homogenate. There was also a trend towards
We next investigated whether the modification of mitochondrial proteins by lipid electrophiles altered mitochondrial function by determining the effect of 15d-PGJ$_2$ and mito-15d-PGJ$_2$ on mitochondrial membrane potential ($\Delta \psi$). MDA-MB231 cells were treated for 4 h with increasing concentrations (1–10 μM) of 15d-PGJ$_2$, or mito-15d-PGJ$_2$, then JC-1 fluorescence was assessed. Treatment with 15d-PGJ$_2$ resulted in a dose-dependent decrease in the mitochondrial membrane potential. However, mito-15d-PGJ$_2$ caused a more significant depolarization of the mitochondrial membrane (Figure 6C). TPMP was used as a control for the effect of the TPP$^+$ moiety and did not cause mitochondrial membrane depolarization.

Measurement of mitochondrial function after exposure to 15d-PGJ$_2$ and mito-15d-PGJ$_2$

To assess cellular bioenergetics in intact MDA-MB231 cells, the Seahorse Bioscience XF24 Extracellular Flux Analyzer was used to determine the rates of change in both pH and oxygen in the medium surrounding the cells [31,39]. We have utilized a mitochondrial function protocol (schematically represented in Figure 7A). This protocol is a modification of one described previously [40]. Initially, basal OCR is measured and, after 20 min, the inhibitor of the mitochondrial ATP synthase, oligomycin (0.3 μg/ml), is added. This causes a decrease in OCR which we ascribe to the mitochondrial activity utilized for ATP synthesis. The remaining OCR can be ascribed to all processes which allow ion movement across the mitochondrial inner membrane, and we collectively term this proton leak. Next, the maximal mitochondrial function that can be sustained in the cells with endogenous substrates is assessed by the addition of the proton ionophore FCCP (1 μM). As expected, this stimulates oxygen consumption, as it is no longer constrained by the proton gradient in the mitochondria. The difference between the basal OCR and this maximal rate is then termed the reserve capacity of the mitochondrial function in the cells under these conditions. Finally, the complex III inhibitor, antimycin A, is added to assess the oxygen consumption of COX, the terminal member of the electron transport chain. The remaining low level of OCR can be ascribed to partial reduction of oxygen and is therefore ascribed to ROS (reactive oxygen species) formation.

Using this protocol, mitochondrial function was measured in confluent MDA-MB231 cells which were first exposed to 10 μM 15d-PGJ$_2$, mito-15d-PGJ$_2$ or mito-PGE$_2$ for 30 min. The lipids were then removed and the mitochondrial parameters measured. There was no significant effect of 15d-PGJ$_2$ or mito-PGE$_2$ on basal or maximal respiration; however, profound decreases in basal and maximal OCR were seen with the mito-15d-PGJ$_2$ treatment (Figures 7B and 7C). The analysis of the different components of OCR, as described above, was also performed and the results are shown in Figure 7(D). It is clear that 15d-PGJ$_2$ has little effect on mitochondrial function, whereas mito-15d-PGJ$_2$ suppresses all bioenergetic parameters. In addition to the measurement of OCR, the ECAR, a surrogate marker of glycolysis, was also determined (Figure 7E). Under unstimulated conditions, ECAR represents predominantly lactate production from glycolysis. Treatment with either 15d-PGJ$_2$ or mito-PGE$_2$ resulted in no change in ECAR; however, after a 30 min exposure to mito-15d-PGJ$_2$, ECAR is significantly increased. It is important to note that at this time point apoptosis has not yet been activated (Figure 7F) thus these mitochondrial bioenergetic defects precede apoptosis. Taken together these results show that mito-15d-PGJ$_2$ modifies mitochondrial function, whereas 15d-PGJ$_2$ has a minimal effect on any of the bioenergetic parameters assessed. Moreover, glycolytic...

![Figure 6](image)

**Figure 6** Localization of 15d-PGJ$_2$ and mito-15d-PGJ$_2$ to the mitochondrion and effects on mitochondrial membrane potential

(A) MDA-MB231 cells were treated with 10 μM bt-15d-PGJ$_2$ (bt-15d) or mito-15d-PGJ$_2$ (Mito-15d) for 1 h then mitochondrial and cytosolic fractions were prepared and resolved using SDS/PAGE (10% gels). Adduct formation was detected using an anti-TPP$^+$ antibody (for mito-15d-PGJ$_2$) or streptavidin–HRP (for bt-15d-PGJ$_2$). ECL$^+$ coupled to HRP was used to detect chemiluminescence protein adducts. VDAC protein levels were also determined using Western blot analysis (lower panels) to confirm mitochondrial preparation purity. (B) Protein adducts were quantified and expressed as the fold-enrichment over the homogenate. EtOH, ethanol vehicle control. (C) MDA-MB231 cells were exposed to increasing concentrations (1–10 μM) of 15d-PGJ$_2$, mito-15d-PGJ$_2$ or TPMP for 4 h, then the mitochondrial membrane potential was assessed using JC-1. Results are represented as the ratio of red/green fluorescence and are means ± S.E.M, n = 3–6. *P < 0.05 compared with EtOH. **P < 0.01 compared with EtOH. ***P < 0.005 compared with 15d-PGJ$_2$.

an increase in mitochondrial protein adducts with bt-15d-PGJ$_2$ treatment; however, this result was not significant.
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Figure 7  Effect of 15d-PGJ2, mito-15d-PGJ2 and mito-PGE2 on basal and maximal respiration capacity

MDA-MB231 cells were treated for 30 min with 10 μM 15d-PGJ2, mito-15d-PGJ2 or mito-PGE2 in specialized 24-well Seahorse Biosciences V7 microplates, then bioenergetic function was assessed using the Seahorse Biosciences XF24 Analyzer. (A) A schematic diagram demonstrating the use of specific inhibitors to determine the sites of cellular oxygen consumption is shown. (B) Basal OCR and (C) maximal OCR for the treatments are shown. EtOH, ethanol vehicle control. (D) The proportion of maximal respiration utilized for ATP-linked respiration (ATP-linked), proton leak (Proton), and ROS formation (ROS), and the reserve respiratory capacity (Reserve) was calculated after measurement of the OCR using the Seahorse Biosciences XF24 Analyzer after sequential injections of oligomycin (0.3 μg/ml), FCCP (1 μM) and antimycin A (10 μM) from MDA-MB231 cells treated as described. (E) Basal ECAR was also measured concomitantly to the experiment in (D). The Seahorse Biosciences XF24 Analyzer protocol included 2 min of mixing, 2 min of waiting and 3 min of measurement times for each measurement. (F) Caspase 9 activation was assessed by Western blot analysis of pro-caspase 9 in samples harvested from a parallel plate, treated as described. A representative Western blot is shown. Results represent means ± S.E.M, n = 5. **P < 0.01 compared with EtOH. #P < 0.05 compared with 15d-PGJ2.

The control of redox signalling in cells is now emerging as a key area for our understanding of both the aetiology of disease and the development of novel therapeutics. It is recognized that redox signalling offers potential therapeutic targets; however, the major limitation has been the inability to selectively activate or inhibit a specific pathway [41]. To our knowledge, this is the first demonstration of selective targeting of a redox signalling pathway by modifying an electrophilic lipid to target the mitochondrion. We selected the compound 15d-PGJ2 for these studies because of its recognized potential as a therapeutic agent and its well-established ability to modify proteins and activate cell signalling in both the cytosol and mitochondrion. We have demonstrated that by conjugating the delocalized cation TPP+ to 15d-PGJ2, we are able to enhance its cytotoxicity by promoting apoptosis over necrosis when compared with the parent compound (Figure 2). It is likely that the mechanism through which this occurs is the covalent modification of proteins as the non-electrophilic lipid analogue to mito-15d-PGJ2, mito-PGE2, has no effect on cell death or caspase 9 activation. There are multiple potential mechanisms by which mito-15d-PGJ2 could enhance apoptosis in cells.

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is unlikely that mito-15d-PGJ$_2$ activates PPAR$_\gamma$-dependent cell death as PPAR$_\gamma$ is localized primarily in the cytosol and blocking the carboxy functional group on the lipid leads to loss of its PPAR$_\gamma$-dependent properties [42,43].

As mito-15d-PGJ$_2$ is added to the outside to the cell it will first accumulate in the cytosol, on the basis of the membrane potential across the plasma membrane, and only then will it cross the inner mitochondrial membrane and accumulate in the mitochondrion [22,44]. Consistent with this model, we found that the protein adducts presented a distinct pattern when separated by two-dimensional IEF SDS/PAGE. As expected, the electrophile-responsive proteome for mito-15d-PGJ$_2$ shares some common targets with 15d-PGJ$_2$, but a far greater number of proteins (53 for bt-15d-PGJ$_2$, 22 for mito-15d-PGJ$_2$) are distinct (Figure 5). The fact that mito-15d-PGJ$_2$ has substantially fewer protein targets is consistent with our hypothesis that the sub-proteomes modified by reactive electrophiles are cell-domain-specific. There are, however, some limitations to this analysis of the electrophilic proteome. For example, not all the modified proteins are capable of entering the second dimension of the two-dimensional IEF SDS/PAGE due to precipitation in the isoelectric strip (results not shown). Nevertheless, it is clear from the proteins that are represented that the patterns are distinct. Moreover, as expected, modified proteins were present in both the cytosolic and mitochondrial fractions of cells exposed to both mito-15d-PGJ$_2$, and the untargeted analogue (Figure 6). Fractionation of the cells after exposure to the electrophiles resulted in a greater enrichment of adducted proteins, relative to the unfractionated sample, for mito-15d-PGJ$_2$, compared with 15d-PGJ$_2$. In further support of a selective impact on mitochondrial function by the addition of the TPP$^+$ group to 15d-PGJ$_2$, we found that mito-15d-PGJ$_2$ caused a profound mitochondrial defect in oxidative phosphorylation at concentrations where 15d-PGJ$_2$ had no discernable effect (Figure 7).

There are several potential mechanisms for the increased toxicity of mito-15d-PGJ$_2$. First, mito-15d-PGJ$_2$ may more extensively modify mitochondrial targets of the parental compound 15d-PGJ$_2$. We have reported previously that 15d-PGJ$_2$ modifies components of the mitochondrial permeability transition pore, including ANT (adenine nucleotide translocator), and promotes permeability transition in endothelial cells [20]. There is also the potential that mito-15d-PGJ$_2$ enhances cell death by limiting the cytoprotective response of cells to the electrophilic stress they experience. Nrf2-dependent gene transcription results in an increase in the overall antioxidant capacity of cells and has recently been demonstrated to also initiate anti-apoptotic responses [45]. A last potential mechanism for increased cytotoxicity of mito-15d-PGJ$_2$ maybe through the modification of new mitochondrial protein targets as a result of the targeting of 15d-PGJ$_2$. Our results suggest that the loss of Nrf2-dependent gene transcription does not explain the enhanced cytotoxicity of mito-15d-PGJ$_2$ (Figure 4). Co-incubation with an Nrf2-activating concentration of 15d-PGJ$_2$ does not protect against mito-15d-PGJ$_2$-induced cell death. Moreover, wild-type and Nrf2-null MEFs have similar cytotoxicity profiles, pointing to the fact that Nrf2 is not a key mediator of the cell death phenotype on acute exposure to an electrophile. However, it is important to note that induction of antioxidant defences with low non-toxic concentrations of electrophiles has been shown to be essential for the protection against subsequent oxidative stress [46].

The fact that the loss of Nrf2 signalling does not appear to be a mediator of the enhanced cell-death effects of mito-15d-PGJ$_2$ does not preclude the role of mitochondrial thiol modification as a mechanism. We have demonstrated that both mito-15d-PGJ$_2$ and 15d-PGJ$_2$ localize to the mitochondrion and, in two-dimensional protein adduct formation analyses, we have identified a number of common protein targets for the two compounds, which are probably the cell-death targets (Figures 5 and 6). Interestingly, we also show that mito-15d-PGJ$_2$ has profound effects on mitochondrial bioenergetics that are not observed with untargeted 15d-PGJ$_2$ (Figure 7). These results suggest a potential role for both the extent of modification of common targets and a gain-of-function activity of mito-15d-PGJ$_2$, through the modification of unique mitochondrial protein targets, that results in impaired mitochondrial respiration and may contribute to cell death. Consistent with this idea, our results also demonstrate that the concentrations at which mitochondrial function is impaired are also those which are required to initiate apoptosis.

We reasoned that if mito-15d-PGJ$_2$ was accumulated in the mitochondrion it would be less effective at activating cytosolic signalling pathways. To test this we chose to measure the level of initiation of the EpRE-dependent genes, such as HO-1 or the proteins controlling GSH synthesis, by the electrophilic lipids [37]. We found that the ability of mito-15d-PGJ$_2$ to up-regulate HO-1 is greatly attenuated and for GSH that it is essentially abolished when compared with 15d-PGJ$_2$ (Figure 3).

In the present study, we have demonstrated the feasibility of targeting specific cell responses by targeting a subcellular proteome (the mitochondrion). We successfully targeted the cellular response of cell death and selected against another (antioxidant up-regulation). It is important to note that the addition of the TPP$^+$ moiety to 15d-PGJ$_2$ may have an impact on the electrophile-responsive proteome through mechanisms distinct from mitochondrial targeting. For example, it probably changes the lipophilicity of the parent electrophile and may also provide additional steric factors which may change the reactivity to target proteins. Taken together these results indicate that both the reactivity of the thiol proteome and the physical chemical properties of the electrophile will determine the specific electrophile-responsive sub-proteomes which are modified and thus the biological responses. This has important implications for both the understanding of the basic mechanisms through which electrophiles mediate redox cell signalling and the potential to refine the protein targets of electrophiles through intracellular targeting strategies.

**AUTHOR CONTRIBUTION**

Anne Diers, Ashlee Higdon, Karina Ricart, and Michelle Johnson performed experiments. Anne Diers, Ashlee Higdon, Michelle Johnson, Aimee Landar and Victor Darley-Usmar designed experiments and analysed results. Anupam Agarwal provided MEFs. B. Kalyanaraman provided reagents for synthesis of mito-15d-PGJ$_2$. All authors contributed to the preparation of the manuscript.

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SUPPLEMENTARY ONLINE DATA
Mitochondrial targeting of the electrophilic lipid 15-deoxy-Δ\(^{12,14}\)-prostaglandin J\(_{2}\) increases apoptotic efficacy via redox cell signalling mechanisms

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SUPPLEMENTARY EXPERIMENTAL

Synthesis and characterization of mitochondrially targeted prostaglandins

For mito-15d-PGJ\(_{2}\) synthesis, the reaction mixture consisted of 2 mg of 15d-PGJ\(_{2}\), 2 mg of EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Pierce Chemicals] and 2 mg of 2-amino-ethyl-TPP bromide in 80% acetonitrile/19% ethanol/1% water (by vol.). The reaction was incubated for 18 h at room temperature with constant mixing. The product was purified by reversed-phase HPLC using a C\(_{18}\) Luna column (Phenomenex) with a linear gradient from 10% acetonitrile/0.24% acetic acid/90% water (by vol.) to 95% acetonitrile/5% water (by vol.). Figure S1 shows the HPLC chromatograms for unmodified 15d-PGJ\(_{2}\) and the reaction mixture for 15d-PGJ\(_{2}\) and amino-ethyl-TPP\(^+\). The eluate was monitored at the characteristic absorbance of the cyclopentenone at 306 nm. As can be seen, the parent 15d-PGJ\(_{2}\) elutes as one peak (Figure S1A) with a retention time of 27 min, whereas in the reaction mixture, this peak is not detectable and is replaced with two peaks (Figure S1B, a and b) at 18–20 min. To further identify the products of the reaction, fractions were collected to capture the products in each peak separately and, after extracting the lipid with chloroform, solvent was evaporated under a constant stream of nitrogen gas and the final product reconstituted in ethanol. The products in each peak separately and, after extracting the lipid with chloroform, solvent was evaporated under a constant stream of nitrogen gas and the final product reconstituted in ethanol.

Prostaglandin E\(_{2}\), which is structurally related to 15d-PGJ\(_{2}\), yet is not electrophilic, was used as a control in the present study. We designed and synthesized the mitochondrially targeted derivative of prostaglandin E\(_{2}\) using the same strategy of conjugation to TPP\(^+\) as described above. The mito-PGE\(_{2}\) product was purified by reversed-phase HPLC with a linear gradient from 10% acetonitrile/90% water to 95% acetonitrile/5% water (by vol.). The eluate was monitored at the characteristic absorbance for the TPP\(^+\) moiety at 225 nm for mito-PGE\(_{2}\). Figure S1(C) shows a representative HPLC trace with the predominant peak (a) corresponding to the product. The addition of the TPP\(^+\) moiety was confirmed by ESI–MS, and the concentration was measured by absorbance at 268 nm using the molar absorption coefficient for TPP\(^+\) of 3000 M\(^{-1}\)·cm\(^{-1}\) [1].

Product integrity and purity for the products mito-15d-PGJ\(_{2}\) and mito-PGE\(_{2}\) was confirmed by ESI–MS and tandem MS (Figure S2). Owing to the positive charge conferred by the TPP\(^+\) moiety, mito-15d-PGJ\(_{2}\) is observed at its exact mass [M]\(^+\) with an m/z value of 604.3 (Figure S2A) and mito-PGE\(_{2}\) at 640.3 (Figure S2B). Figure S2(C) shows the tandem MS spectra for mito-15d-PGJ\(_{2}\) and the predicted ion fragments that we propose are derived from the parent structure. We could not detect the uncharged

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fragments in either positive- or negative-ion-mode, presumably due to hydrophobic and uncharged character.

IUPAC nomenclature for the compounds used in the present study is as follows: 15d-PGJ2 (11-oxo-prosta-5Z,9,12E,14E-tetraen-1-oic acid); mito-15d-PGJ2 (11-oxo-prosta-5Z,9,12E,14E-tetraen-1-amido-ethyl triphenyl phosphonium); and mito-PGE2 (9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-amido ethyl triphenyl phosphonium).

UV–visible spectra
To both assess the integrity of the products and determine their concentration, visible absorption spectroscopy was used. To achieve this, the molar absorption coefficient for each wavelength was calculated for 15d-PGJ2 by using a known concentration diluted in ethanol (Figure S3A). The same procedure was performed for TPMP (Figure S3B). The molar absorption coefficient for the compounds at all wavelengths can then be determined and is shown in Figure S3. The simulated spectra of the product, mito-15d-PGJ2, was then generated by adding the molar absorption coefficients calculated for TPMP and 15d-PGJ2, assuming a 1:1 molar ratio of the TPP+ moiety to the lipid. Finally, the simulated spectrum was compared with the actual spectrum obtained using the absorbance spectrum for mito-15d-PGJ2 (Figure S3C) and was found to be essentially identical. Taken together, these results demonstrate that the conjugation at carbon-13 in the parent compound, characteristic of the cyclopentene, is unmodified by the addition of amino-ethyl-TPP+. In subsequent experiments, the concentration of mito-15d-PGJ2 was therefore measured by absorbance at 306 nm using a molar absorption coefficient at 306 nm of 12000 M⁻¹·cm⁻¹ [2].

Mitochondrial fractionation
Following experimental incubations, MDA-MB231 cells were harvested by scraping in ice-cold HMIM buffer (20 mM Tris/HCl, pH 7.35, 300 mM sucrose and 2 mM EGTA, at 4°C) containing protease inhibitor cocktail (Roche) and 1 mM PMSF. Cell suspensions were then homogenized used 18 passes with a Potter–Elvehjem homogenizer powered by a drill press [3]. Intact cells and nuclei were removed by centrifuging the sample at 1000 g for 10 min at 4°C. The supernatant containing the mitochondria was then centrifuged at 15000 g for 10 min at 4°C. The supernatant from this step were removed and aliquoted as the cytosolic fraction. The mitochondrial pellet was washed with 1 ml of HMIM buffer and centrifuged again at 15000 g for 10 min at 4°C. The mitochondrial pellet was resuspended in 40 μl of HMIM buffer and protein measurements for all fractions were measured using the Lowry method. Citrate synthase activities were measured using standard spectrophotometric methods as described previously [4,5].

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Figure S3  UV–visible spectra of 15d-PGJ₂ and mito-15d-PGJ₂

Representative UV–visible spectra of 15d-PGJ₂, the TPMP moiety and the synthesized compound mito-15d-PGJ₂. The molar absorption coefficient for each compound was calculated using measured absorbances as described in the Supplementary Experimental section. Molar absorption coefficient against wavelength for (A) 15d-PGJ₂, (B) TPMP and (C) the simulated (red trace) superimposed on the measured spectra for mito-15d-PGJ₂ are shown.
Figure S4 Validation of the mitochondrial fractionation procedure

Mitochondrial fractions from MDA-MB 231 cells were prepared as described in the Supplementary Experimental section. (A) VDAC (an outer mitochondrial membrane protein) and COX subunit I (an inner mitochondrial membrane protein) levels were determined in homogenate, mitochondrial and cytosolic fractions using Western blot analysis. Equivalent amounts of protein were loaded for each sample and equal protein loading was confirmed after transfer on to nitrocellulose membranes using Ponceau S staining. (B) Quantification of the Western blots and the activity of the mitochondrial matrix enzyme citrate synthase are shown. Values represent means ± S.E.M., n = 3. **P < 0.01 compared with the homogenate.

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