Upregulation of Human Prostaglandin Reductase 1 (PTGR1) Improves Efficacy of Hydroxymethylacylfulvene, an Anti-tumor Chemotherapeutic Agent

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Human PTGR1 activates acylfulvenes and improves efficacy

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Abbreviations:
AF, acylfulvene; AOR, alkenal/one oxidoreductase; ARE, antioxidant response element; D3T, 1,2-dithiol-3-thione; HMAF, hydroxymethylacylfulvene; Nrf2, nuclear factor-like 2; PTGR1, prostaglandin reductase 1; rPTGR1, rat PTGR1; hPTGR1, human PTGR1; PCR, polymerase chain reaction; AFs, acylfulvenes; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; NQO1, NAD(P)H:quinone oxidoreductase; CYP450, cytochrome P450; GEDPT, gene-directed enzyme prodrug therapy; cDNA, circular DNA; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline; DTT, dithiothreitol.

Recommended section assignment: Chemotherapy, Antibiotics, and Gene Therapy; Metabolism, Transport, and Pharmacogenomics; Toxicology
Abstract

Prostaglandin reductase 1 (PTGR1) is a highly inducible enzyme with enone reductase activity. Previous studies demonstrated the role of rat PTGR1 in the activation of acylfulvene analogues, a class of antitumor natural product derivatives. Of these, hydroxymethylacylfulvene (HMAF) was in advanced clinical development for the treatment of advanced solid tumors, including prostate, ovarian and pancreatic cancers. However the efficiency of human PTGR1 in activating acylfulvenes, or potential to enhance therapeutic efficacy has remained uncharacterized. In this study, human PTGR1 was PCR-cloned and purified. Conversion of HMAF to its cellular metabolite by the purified enzyme proceeded at a twenty-fold higher rate than with the rat variant of the enzyme. The $K_m$ was 4.9 $\mu$M, forty-fold lower than for the rat variant and similar to the therapeutic dose. Human cell lines, including colon cancer lines, were transfected with a vector containing rat PTGR1 or human PTGR1, and cell viability was examined after dosing with HMAF. New data obtained in this study suggests that transfection with human PTGR1, or its induction in colon and liver cancer cell lines with 1,2-dithiol-3-thione, enhances susceptibility to the cytotoxic influences of HMAF by 2–10 fold. Furthermore, similar or enhanced enzyme induction and HMAF toxicity results from preconditioning cancer cells with the bioactive food components curcumin and resveratrol. The functional impact of PTGR1 induction in human cells and chemical-based strategies for its activation can provide important knowledge for the design of clinical strategies involving reductively activated cytotoxic chemotherapeutics.
Introduction

Chemotherapeutic agents, together with surgery, radiation and immunotherapy, are key components of effective modern anticancer therapy. Among them, alkylating agents are highly effective tumor cytotoxins. These drugs elicit therapeutic responses by covalently binding to cellular nucleophiles, such as DNA and proteins, thereby leading to stalled DNA synthesis, inhibited enzyme function, and tumor cell death. Several classes of alkylating agents, including nitrogen mustards (i.e. cyclophosphamide and ifosfamide) and quinones (i.e. mitomycin C) gain a selective advantage in tumor cells on the basis of enzymatic bioactivation that unmasks chemical electrophiles (Powis, 1983). A detailed understanding of the chemical and biochemical mechanisms of bioactivation, including the identification of specific drug metabolizing enzymes in human cells, can improve drug design and clinical therapeutic strategies.

Acylfulvenes (AFs) are semisynthetic derivatives of the mycotoxin illudin S (1, Scheme 1) (Kelner et al., 1996; McMorris et al., 1996). Illudins (1, 2, Scheme 1) are potent cytotoxins at nanomolar concentrations in several human cancer cell lines (Kelner et al., 1997), but have poor therapeutic indices and are associated with substantial systemic cytotoxicity in animal models (Kelner et al., 1987). Acylfulvene (AF, 4, Scheme 1) and its analogues, including clinically tested hydroxymethylacylfulvene (HMAF, marketed as irofulven 3, Scheme 1), are up to two orders of magnitude less cytotoxic than illudin S, but retain efficacy in tumor cell lines and xenografts (MacDonald et al., 1997; Gong et al., 2006). Outcomes from clinical trials with HMAF were mixed, ranging from little effects in non-small cell lung cancer and gastric cancer to modest effects in ovarian and pancreatic cancer. The array of side effects included thrombocytopenia, nausea and
vomiting, visual symptoms and renal dysfunction (Tanasova and Sturla, 2012). These observations suggest a pressing need to better understand factors controlling toxicity with the aim of devising improved analogs or therapeutic strategies.

The metabolic enzyme prostaglandin reductase 1 (PTGR1)\(^1\) has been invoked as a key activating enzyme that significantly influences the activities of AF analogues (Dick et al., 2004; Gong et al., 2006; Gong et al., 2007). PTGR1 was originally identified as leukotriene B4 dehydrogenase (LTBDH4) (Yokomizo et al., 1993), and it transforms prostaglandins by enone reduction (Tai et al., 2002; Yu et al., 2006). PTGR1 was later discovered to be strongly induced by 1,2-dithiol-3-thione (D3T) in rat liver by NF-E2-related Factor 2 (Nrf2)-mediated gene activation (Primiano et al., 1996; Dick et al., 2001), and further characterized as alkenal/one oxidoreductase (AOR) for its capacity to reduce endogenous and exogenous \(\alpha,\beta\)-unsaturated aldehydes and ketones (Dick et al., 2001; Dick and Kensler, 2004; Gong et al., 2006; Yu et al., 2006; Gong et al., 2007; Neels et al., 2007).

Rat PTGR1 (rPTGR1) was shown to be a determining factor in the activation of AFs \textit{in vitro} (Dick et al., 2004), however, the competence of human PTGR1 (hPTGR1) in HMAF bioactivation has not been defined. The primary sequences of rPTGR1 and hPTGR1 are 83% identical and 329 amino acids in length. Primary sequence differences include the NADPH binding domain (Supplemental Figure 1; (Hori et al., 2004). With NADPH as cofactor and AF analogs as substrates, enone reduction at the 8,9-double bond primes the AF cyclopropane for nucleophilic ring-opening and results in aromatization of the six-member ring (\(5\), Scheme 1) (Dick et al., 2004). Ectopic expression of rPTGR1 in human embryonic kidney cells (HEK293) reduced the IC\(_{50}\) of
HMAF 100-fold. Furthermore, reductase activity in NCI-60 cell line extracts correlates with their sensitivity to HMAF (Dick et al., 2004), but there is no direct information available regarding the bioactivation of HMAF by hPTGR1. Data obtained in this study establishes the role of hPTGR1 in HMAF bioactivation and explores strategies to sensitize human tumor cells to the drug.

In response to a wide range of endogenous and exogenous signals, many enzymes are regulated by the transcription factor Nrf2, a central regulator in xenobiotic metabolism and the cellular stress response. Nrf2 triggers include thiol-modifying chemicals like dithiolthiones and sulforaphane, and Michael-type acceptors such as triterpenoids and retinoid acid derivatives, among others (Figure 1) (Kwak et al., 2004). Reactions with cysteine residues on the protein Keap1, a negative regulator of Nrf2, lead to Nrf2 translocation from the cytoplasm into the nucleus. Coupled with cofactors in the nucleus, Nrf2 recognizes the antioxidant response element (ARE) in the promoter regions of its target genes and initiates transcriptional activation (Kwak et al., 2001). This molecular mechanistic paradigm offers possible options to modulate PTGR1 expression in human cells and, in addition, carries implications for potential anticancer therapies involving a PTGR1 inducer in combination with bioreductive drugs.

Materials and Methods

Chemicals and reagents. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. All solvents and chemicals were of analytical reagent grade or higher. HMAF was provided by MGI Pharma, Inc. (now part of Eisai Co., Japan).
Plasmid construction. hPTGR1 was amplified by polymerase chain reaction (PCR) from a human adult liver cDNA library (Cat# 7113-1, Lot# 1040535, Clontech, Mountain View, CA), according to the NCBI GenBank sequence (Accession# NM_012212) using the following primer set:

Forward: 5’-GTCGCGGAATTCAGCTTCAGGATGGTTCGTACTAAGACATGG,
Reverse: 5’-GTCGCGCTCGAGTTACTATCATGCTTTCACTATTGTCTTCCCC.

PCR product was cleaned and ligated into pBlueScript (Agilent, Santa Clara, CA) between EcoRI and XhoI sites. DNA insert was confirmed by sequencing and subcloned into episomal vector pCEP4.

For N-terminal FLAG-tagged recombinant protein expression, both rPTGR1 and hPTGR1 were PCR cloned using following primer sets to omit the starting ATG codon:

rPTGR1 forward, GCGAATTCAGTACAAGCTAAGACCTGGAC; rPTGR1 reverse, GCTCTAGATCACGCTTTCACTATAGTCTTCC; hPTGR1 forward, GCGAATTCAGTTCGTACTAAGACATGGAC; hPTGR1 reverse, GCTCTAGATCATGCTTTGACTATTGTCTTCC. PCR products were cleaned and ligated into p3×FLAG-myc-CMV-24 (Sigma-Aldrich, St. Louis, MO) at EcoRI and XbaI sites to produce N-terminal FLAG tagged proteins. DNA insert was confirmed by sequencing.

Cell culture and transfection. HEK293, HCT15, SW620 and HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Plasmids were
transfected into cells with Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations (http://tools.invitrogen.com/content/sfs/manuals/lipofectamine2000_man.pdf).

**Recombinant protein expression and purification.** rPTGR1 was expressed in *E. coli* as previously described (Dick et al., 2004). p3×FLAG-hPTGR1 was transiently transfected into HEK293 cells as described above. Cells were harvested 24 h later and resuspended in lysis buffer (50 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100) at a concentration of 1.5 × 10^7 cells/mL buffer. Cell lysate was centrifuged for 15 min at 12000g to eliminate cell debris. Supernatant was incubated with anti-FLAG agarose gel (Sigma-Aldrich, St. Louis, MO) at a ratio of 1 mL per 200 μL gel and mixed for 2 h at 4 °C. Agarose gel was washed with 5 bed volumes of Tris buffer (50 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl) 3 times and eluted 3 times with 1 bed volume wash buffer containing 500 μg/mL 3×FLAG peptide. The eluent was dialyzed against 25 mM Tris buffer (pH 7.4, 1 mM dithiothreitol (DTT)) at 4 °C overnight and aliquots were adjusted to 10% glycerol and stored at -80 °C.

**Cell viability assay.** pCEP4, pCEP4-rPTGR1 and pCEP4-hPTGR1 transfected HEK293 cells were maintained in DMEM supplemented with 10% FBS and 100 μg/mL hygromycin B (Invitrogen, Carlsbad, CA). Cells were plated in 96-well culture plates 18 h prior to treatment at a density of 4000 cells/well. Toxicity studies were conducted by replacing medium with the complete medium containing HMAF (stock solutions prepared in 100% dimethylsulfoxide (DMSO)) at desired concentrations. Cell viability was measured 24 h later by MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Invitrogen). HCT15, SW620 and HepG2 cells were
maintained in DMEM with 10% FBS. pCep4-GFP or pCep4-PTGR1-transfected HCT15 cells were seeded in 96-well plates at 8000 cells/well, 6 h prior to treatment. Toxicity studies were conducted as mentioned above. Cell viability was measured 18 h later via Cell Titer-Blue assay (Promega, Fitchburg, WI). SW620 and HepG2 cells were incubated with 20 μM D3T, 10 μM curcumin or 15 μM resveratrol in DMEM+10% FBS for 48 h prior to seeding in the 96-well plates. After washing out inducers with 1X phosphate buffered saline (PBS), cytotoxicity assay was carried out as described for HCT15 cells. Survival fraction of each cell population was calculated by normalizing against the vehicle-treated population within each experimental arm. IC₅₀ values were calculated via mathematical simulations of non-linear regression based on data obtained from three or more independent experiments. Regression and statistical analyses were performed using SigmaPlot (version 10.0).

**Enzyme kinetic studies.** Due to the low yield of hPTGR1 from mammalian cells compared to rPTGR1 from bacterial cells, a very conservative protocol needed to be established for hPTGR1. In the process of developing the protocol for hPTGR1, rPTGR1 was used to assess the method and results highly comparable to the standard rPTGR1 protocol were obtained. Initial velocities were measured by monitoring the decay of the absorbance at 420 nm (2210 cm⁻¹ M⁻¹ for HMAF) (Gong et al., 2006) at 37 °C with a Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA) or a Beckman DU 800 spectrophotometer (Beckman Coulter, Indianapolis, IN). Stock solutions of analytes were prepared in DMSO (50 mM) and were diluted to a final concentration between 0.05 and 0.4 mM. The percentage of DMSO in each experiment was below 0.2% of the total volume. A saturating concentration of NADPH was used.
(150 μM) in 100 μL of reaction buffer (0.5x PBS supplemented with 0.01% Triton X-100, 0.05% bovine serum albumin) with 1 μg recombinant enzyme for hPTGR1 and for rPTGR1 in 1 mL of reaction buffer with 5.1 μg recombinant enzyme. 100 μg cell lysates were used to calculate the reaction rate for transfected cells. The UV-vis absorbance spectrum was recorded for 4 min. $K_m$ and $V_{max}$ were calculated using SigmaPlot (version 12.2).

**Promoter analysis.** Consensus sequences for transcription factor binding sites in the promoter region of PTGR1 were analyzed with Genomatix Gene2promoter software (www.genomatix.com). Sequences of PTGR1 with experimentally verified 5’ transcripts from database ElDorado 12-2010 were utilized. Up to 1000 base pairs upstream of the transcription starting site was analyzed and consensus transcriptional factor binding sites were identified.

**Immunoblot analysis.** Cultured cells were seeded in 6-well culture plates at 1 × 10⁶ cells/well 18 h before treatment with 20 μM D3T, 10 or 20 μM curcumin, 15 or 40 μM resveratrol, or DMSO (vehicle control) in the growth medium. After 48 h preconditioning, cells were washed with ice-cold 1 x PBS and collected by centrifugation following trypsinization. After seeding for viability assay, the remaining cells were resuspended in RIPA Buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). The suspension was vortex mixed and lysate collected by centrifugation at 12000g. Protein extracts were analyzed by SDS-PAGE and electrotransferred to a nitrocellulose membrane with iBlot (Invitrogen, Carlsbad, CA). Immunodetection was performed using mouse anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) and anti-PTGR1 antibody (LTB4DH polyclonal antibody, cat# H00022949-A01, Abnova, Walnut,
CA,) followed by horseradish peroxidase-conjugated rabbit-anti-mouse serum (Pierce, Hercules, CA). Electrochemiluminescence reagents (Amersham, Piscataway, NJ) were used for chemiluminescent detection. Densitometry analyses were performed using ImageJ software.

**Quantitative RT-PCR.** Total RNA was purified using Trizol Reagent (Life Technologies, Carlsbad, CA). Total RNA (1 μg) was reverse transcribed in a 50 μL reaction volume with TaqMan Reverse Transcription Reagents (Applied Biosystems Inc. Foster City, CA). A 5 μL solution of the reverse transcribed cDNA was PCR amplified according to Applied Biosystems recommendations ([http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf](http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf)). TaqMan probes were purchased from Assay-on-Demand (Applied Biosystems Inc., Carlsbad, CA). Three replicate reactions were run for each RNA sample.

**Statistical analysis.** Statistical comparison was performed using pairwise t-test for single pairs and ANOVA for multiple groups using SigmaPlot (version 10.0).

**Results**

**Cloning and expression of hPTGR1.**

hPTGR1 was PCR-amplified from a human adult liver cDNA library according to the NCBI GenBank sequence. The recombinant expression was not successful in modified *E. coli* strain BL21/codon+ or *Drosophila* cell line S2. Therefore, protein expression was carried out in HEK293 cells, which were selected for their high transfection efficiency. Plasmid containing 3×FLAG tagged hPTGR1 was transiently transfected and the cells were allowed to grow for additional 48 h. Cell lysate was
purified by affinity tag techniques and analyzed by gel electrophoresis with hPTGR1 and FLAG antibody (Supplemental Figure 2). Further, the eluted hPTGR1 was verified to be pure by Coomassie staining. Typical yields are 150 μg of recombinant hPTGR1 per 1 × 10^8 cells.

**hPTGR1 activates HMAF and improves drug efficacy.**

With the successful expression and purification of hPTGR1, it was possible to directly address the competence of the enzyme in catalyzing the bioactivation of HMAF. Kinetic parameters of hPTGR1-catalyzed HMAF metabolism were measured and the comparison between performances of rPTGR1 versus hPTGR1 is shown in Table 1. hPTGR1 was more efficient than rPTGR1. The 20-fold higher $V_{max}/K_m$ value is largely attributable to a greater than 40-fold lower $K_m$ for HMAF, and a similar catalytic rate.

HEK293 cells transfected with blank episomal vector pCEP4, rPTGR1-expressing vector pCEP4-rPTGR1, or hPTGR1-expressing vector pCEP4-hPTGR1 were challenged with graded concentrations of HMAF and cell viability was determined by MTT assay. Data obtained from three independent studies (Figure 2) demonstrated that both rPTGR1 and hPTGR1 transfection enhanced the cytotoxicity of HMAF more than 15-fold relative to the vector control (IC$_{50}$ ~1 μM). In accordance with the enzymatic data, HMAF consumption by total cell lysate from hPTGR1-transfected cells exhibited a more than 14-fold higher conversion rate, 247.6 versus 17.5 nmole/min/mg (Table 2). Consequently, hPTGR1 was more effective at sensitizing cells to HMAF, further reducing the IC$_{50}$ to 25 nM from 54 nM in rPTGR1 transfected cells (Table 2, $P < 0.05$). These observations regarding a higher kinetic proficiency of hPTGR1 in a cell-free system, greater
conversion by total cell lysate, and associated enhanced cellular sensitivity to HMAF are in accordance with a role for hPTGR1 in HMAF bioactivation.

**Overexpression of hPTGR1 in colon cancer cell line HCT15 enhances HMAF cytotoxicity.**

The results presented above involving HEK293 cells provided a proof-of-concept regarding the role of hPTGR1 in activating HMAF. We further examined the hypothesis that hPTGR1 upregulation is associated with higher HMAF toxicity in more clinically relevant cancer cell models. Thus, an hPTGR1- or GFP-containing vector was transiently expressed in the colon cancer cell line HCT15. After 48 h, transfected cells were challenged with graded concentrations of HMAF. Ectopic expression of hPTGR1 was 1.9-fold higher than the endogenous level, as determined by densitometry following immunoblots (Table 3, Supplemental Figure 3a). The PTGR1 level increase was modest, but may be more physiologically relevant than the typical 25–100-fold increases of PTGR1 observed in transfected HEK293 cells (Dick et al., 2004). Moreover, the modest increase of hPTGR1 protein level reduced the IC\(_{50}\) value of HMAF, from 16.9 μM to 5.7 μM (\(P < 0.05, n = 3, \)Figure 3, Table 3). This observation demonstrated for the first time that HMAF cytotoxicity can be enhanced by over-expression of hPTGR1 in a human cancer cell line.

**Pretreatment of D3T increases HMAF cytotoxicity.**

PTGR1 was discovered in a differential screening of D3T-treated rat liver, associating it with Nrf2-mediated gene activation (Primiano et al., 1996). Sequence analyses by Genomatix gene2promoter algorithm revealed multiple Nrf2 binding sites 1000 base pairs upstream of the transcription starting site in both variants of PTGR1.
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(Figure 4a). Thus, colon cancer cell line SW620 was incubated for 48 h with 20 μM D3T before cell lysate was analyzed. Immunoblotting showed, as determined by densitometry, a 2-fold upregulation of PTGR1 (Supplemental Figure 4b). Furthermore, when these preconditioned cells were treated with HMAF, there was a reduction of IC₅₀ value to 9.9 μM compared to 20.1 μM for vehicle-treated cells (P < 0.05, Figure 4b, Table 4).

Hepatocytes are well established models for ARE-mediated enzyme inductions (Otieno et al., 2000). PTGR1 was previously shown to be highly inducible in rat liver by D3T (Primiano et al., 1996). Therefore the hepatocarcinoma cell line HepG2 was selected to test the impact of D3T preconditioning on HMAF toxicity. After incubation with D3T for 48 h, cells were washed and then subjected to HMAF at increasing concentrations and viability was measured 18 h after HMAF treatment. Indeed, the IC₅₀ value, which decreased from 31 μM to 3.0 μM, was more significantly reduced than it was for the colon cancer cells (Figure 4b). Immunoblot analysis indicated 3-fold PTGR1 induction, also higher than for the colon cells, consistent with a positive correlation between PTGR1 levels and drug efficacy (Table 4, supplemental Figure 4).

**Curcumin and resveratrol upregulate hPTGR1 in HepG2 and SW620 cells, leading to enhanced HMAF toxicity.**

Extensive data supports the effectiveness of plant-based polyphenols, including dietary components such as curcumin (Figure 1), a polyphenol in the spice turmeric, and resveratrol (Figure 1), a stilbenoid in grape skins, in activating cytoprotective enzymes (Birringer, 2011). Such bioactive food components may be convenient, accessible, and efficient options for modulating desired enzymatic activities. RNA (24 h) and protein levels (48 h) of PTGR1 were examined in HepG2 and SW620 cells after treatment with
10 and 20 μM curcumin or 15 and 40 μM resveratrol. Compared to 20 μM D3T, these compounds similarly elevated PTGR1 levels (Figure 5a). At 20 μM, curcumin was even more effective than the same dose of D3T in SW620 cells, elevating the RNA level of PTGR1 5-fold. Immunoblot indicated that the upregulation at the protein level correlated well with mRNA quantitation (Supplemental Figure 4).

Effects of pretreatment on the toxicity of HMAF were examined after HepG2 and SW620 cells were incubated with 20 μM D3T, 10 μM curcumin or 15 μM resveratrol for 48 h. With the exception of SW620 cells with 10 μM curcumin, all other pretreatments significantly enhanced cytotoxicity of HMAF and lowered the IC50 by 2–7 fold (Figure 5b, Table 4).

Discussion

rPTGR1 has been previously expressed in *E. coli* as a recombinant protein. Difficulties in expressing the human enzyme in this system may be due to the abundance of proline residues within the 3′ coding region of hPTGR1. Proline is an infrequently utilized amino acid in *E. coli*, accounting for 4% of total amino acids. Three of the four coding codons, CCC, CCU and CCA, occur at an extremely low frequency of 3.5 to 7.5 per 1000 codons (Sharp et al., 1988). The presence of a proline-rich sequence “PLPPGPPP” within hPTGR1 (amino acid 250-257, Supplemental Figure 1) and the corresponding coding sequence, “CCA CTT CCC CCA GGC CCA CCC CCA,” that features six rare codons in close proximity, is likely to stall *E. coli* translation machinery, leading to the degradation of the partially translated protein. While mutation of CCC and CCA codons to CCG, the proline codon used four times more frequently in *E. coli* (Sharp
et al., 1988), may help to circumvent this obstacle; in the present study we successfully addressed the problem by expressing the protein in human cells.

An aim of this study was to examine the role of hPTGR1 in HMAF activation based on the understanding that rPTGR1 overexpression was previously shown to greatly sensitize HEK293 cells towards AFs. In fact, we discovered that hPTGR1 exhibited an even higher catalytic activity towards HMAF compared to rPTGR1, leading to a higher cytotoxicity in HEK293 cells (Figure 2). In vitro studies indicated that this enhancement is largely due to the more than 40-fold lower $K_m$ of hPTGR1 towards HMAF (4.9 μM for hPTGR1 versus 215.1 μM for rPTGR1, Table 1). IC$_{50}$ values of therapeutic HMAF concentrations tested in a NCI-60 cell line screen ranged between 0.2–80 μM (data provided by developmental therapeutic program at NCI). Therefore, hPTGR1 should have a profound impact on the activation of HMAF in vivo, since the lower $K_m$ value would allow hPTGR1 to operate at high efficiency within the drug’s therapeutic window. As indicated in Table 2, cell lysate from HEK293 cells overexpressing hPTGR1 versus rPTGR1 displayed a much higher conversion rate of HMAF. Furthermore, a small increase in hPTGR1 level (2-fold in Table 3, Supplemental Figure 3) resulted in a three-fold HMAF IC$_{50}$ reduction in HCT15 cells (Figure 3, Table 3). Since one of the hurdles in multiple HMAF clinical trials was systemic toxicity, bioactivation by hPTGR1 could markedly lower the therapeutic dose of HMAF, and therefore preserve efficacy while ameliorating unwanted side effects.

Like HMAF, many chemotherapeutic agents are administered as a pro-drug, transformed in vivo by enzymatic bioactivation into proximal reactive species. Levels of drug-metabolizing enzymes can have pronounced effects on efficacy of therapeutics.
Nitrosamine drugs can be significantly enhanced by increasing the level of cytochrome P450 (CYP450) reductase. In both aerobic and hypoxic conditions, tiraparamine (SR4233) and RSU 1069 were shown to be two- to ten-fold more effective in multiple breast cancer cell lines when exogenous CYP450 reductase was introduced. Accordingly, drug IC₅₀ values correlate with the expression of CYP450 reductase in tested primary tumors (Patterson et al., 1995a; Patterson et al., 1995b). Recently, overexpression of aldo-keto reductase 1c3 in HCT116 cells enhanced PR104A cytotoxicity by 10-fold, and 1c3 expression correlated with drug sensitivity, as demonstrated by screening of tissue arrays (Guise et al., 2010).

Various strategies have been explored to modulate the level of drug-activating enzymes to enhance efficacy, such as gene-directed enzyme prodrug therapy (GDEPT) and subsequent diffusion of active metabolites into the solid tumor and its microenvironment (Roy and Waxman, 2006). However, limited efforts have focused on pharmacological means for enhancing activating enzymes, such as bioactive food components (Tanasova and Sturla, 2012). A series of studies by Begleiter and coworkers supports the feasibility of the approach; and many relevant enzymes with ARE element in their promoter region can be activated via the Nrf2 pathway (Kwak et al., 2004). Therefore, by using a chemical inducer such as D3T, efficacy may be enhanced by a strategy that would be less invasive than GDEPT. For example, mitomycin C and EO9 efficacies were greatly enhanced in a number of cancer cell lines by elevating NQO1 with D3T pretreatments of 50–100 μM (Doherty et al., 1998; Wang et al., 1999). Furthermore, such enhancement was also observed in vivo when mice were treated with the inducer dimethyl fumarate (Begleiter et al., 2004; Digby et al., 2005).
Similar to NQO1, PTGR1 was discovered to be highly inducible in rat liver by D3T (Primiano et al., 1996). The presence of Nrf2 binding sites within the PTGR1 promoter region (Figure 4a) suggests activation by Nrf2-dependent inducers. In this study, pretreatment of SW620 and HepG2 cells with 20 μM D3T elevated PTGR1 levels (Supplemental Figure 4b) and led to enhanced sensitivity to HMAF (Figure 4b). Even more intriguingly, curcumin and resveratrol both demonstrated comparable or even greater enhancement of PTGR1 upregulation (Figure 5, Supplemental Figure 4) as supported by RNA and protein levels. More importantly, preconditioning with 10 μM curcumin significantly enhanced HMAF cytotoxicity in HepG2 cells and 15 μM resveratrol in both HepG2 and SW620 cells (Figure 5b, Table 4).

There is a lack of response of SW620 cells to HMAF following pretreatment with 10 μM curcumin, suggesting that other cellular processes, such as DNA repair (Tanasova et al., 2012), could contribute to HMAF resistance. It has been reported that activation of DNA repair pathways could lead to a diminished toxicity effect at higher drug doses in target cell population (Smith and Grisham, 1983). Such repair induction may account for some features of the survival curves measured in the present study, such as the slight plateau in the mid-dose range of HCT15 cell survival curves (Figure 3). The DNA-alkylating nature of HMAF may lead to activation of DNA repair once its concentration has reached a critical threshold. The dampened cytotoxic effect is observed in both control and PTGR1-transfected HCT15 cells, therefore, it does not appear to be PTGR1 related. Additionally, curcumin- and resveratrol-treated cells responded in a somewhat biphasic fashion (Figure 5b), such that there was an attenuation of toxicity response, again, in the mid-range of dosage, similar to that observed for HCT15 cells (Figure 3).
On the basis of features of the survival curves that suggested modest biphasic behavior for curcumin- or resveratrol-treated cells, we further analyzed the toxicity data in two separate phases. Independent linear regression analyses of four lowest- and four highest-dose data points suggest that the pre-conditioned cells were significantly more sensitive to HMAF in the low-dose regime in all 3 experiments. For the high-dose regime, a similar differential response is apparent, however only in two of three independent experiments. Such observation underscores the complexity of cellular response to toxins, and that multiple independently regulated pathways can influence toxicity under different conditions. Furthermore, such dose-modulated responses by HMAF seem to be cell-line-specific.

Previous reports suggest that for the precursor illudins, preferential killing of tumor cells could result from increased cellular transport (Kelner et al., 1990); however, for HMAF preferential accumulation did not significantly account for enhanced toxicity in PC-3 and HT-29 cells (Woynarowska et al., 2000). Furthermore, microarray studies regarding cellular responses to D3T or resveratrol have failed to implicate alterations in membrane transport (Kwak et al., 2003; Whyte et al., 2007). Despite these data, transport and repair have not been explicitly evaluated in this study and cannot be excluded as possible factors contributing to cytotoxicity. Thus, although our data suggest that HMAF cytotoxicity can be enhanced using dietary supplements to upregulate hPTGR1, more studies are needed to elucidate the feasibility of such an approach.

Two additional factors relevant to the metabolic induction as a sensitization strategy concern the selectivity of PTGR1 upregulation in target tissues, and the physiological relevance of inducer concentrations. In the present study, D3T was
effective in HepG2 and SW620 cells, but not in HCT15, HCT116 or AGS cells (data not shown). Previous animal studies demonstrated that D3T induced ARE response genes in mouse forestomach, liver, colon and brain (Ramos-Gomez et al., 2001; Burton et al., 2006; Osburn et al., 2007). Thus, it would be informative, in future studies, to profile the modulation of human PTGR1 in various tissues following curcumin or resveratrol treatment. Finally, the concentrations of curcumin and resveratrol used in this study are relatively low compared to other cellular studies commonly appearing in the literature, and moreover, in vivo data suggests that 10–20 μM curcumin and resveratrol may be achieved in the cells of human patients (Garcea et al., 2005; Boocock et al., 2007).

On the basis of preclinical data, drug bioactivation is a significant controlling factor for HMAF cytotoxicity and selectivity. However, mechanistic details regarding human physiological conditions remains to be elucidated. The data obtained in this study suggest that hPTGR1 effectively transforms HMAF into its active form at therapeutic concentrations and enhances its efficacy in cell-based models. Furthermore, data indicates that modulating hPTGR1 is feasible in vitro by food components. These observations establish a proof-of-principle regarding hPTGR1 in cytotoxicity and for clinical implementations of HMAF or related chemotherapeutic agents.

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Conducted experiments: Yu, Erzinger, Cervoni-Curet, Whang

Contributed new reagents or analytical tools: Yu, Pietsch

Performed data analysis: Yu, Erzinger, Cervoni-Curet, Whang

Wrote or contributed to the writing of the manuscript: Yu, Pietsch, Erzinger, Niederhuber, Sturla
References


Footnotes

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PTGR1, prostaglandin reductase 1, accession # 012212 in National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov), was previously reported under the name Leukotriene B4 reductase (LTB4DH), Dithiol-thione inducible gene 1 (DIG1) and alkenal/one oxidoreductase (AOR) in others and our studies.
Figure Legend

Scheme 1. Bioactivation of acylfulvenes by PTGR1. The structures of illudin M and S, HMAF and AF are shown (1–4). Reduction at the C8 position results in the unstable cyclohexadiene intermediate 5, which may react with water or other cellular nucleophiles (R³).

Figure 1. Structures of D3T (dithiol-3-thione), and the dietary components curcumin ((1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) and resveratrol (3,4’,5-Stilbenetriol), three examples of Nrf2 triggers used in this study.

Figure 2. hPTGR1 markedly sensitized HEK293 cells towards HMAF. HEK293 cells transfected with pCEP4, pCEP4-rPTGR1 or pCEP4-hPTGR1 were challenged with HMAF at indicated concentrations. Cell viability was assessed 24 h later. Data were obtained from three independent experiments; error bars represent standard error.

Figure 3. Overexpression of hPTGR1 in colon cancer cell line HCT15 significantly enhances HMAF cytotoxicity. Typical survival graph is shown above, error bars represent standard deviation. IC₅₀ was calculated from three independent experiments by non-linear regression analysis (p < 0.05).

Figure 4 a. Identification of potential Nrf-2 binding sites (indicated as 🌸) by promoter sequence analysis 1000 base pairs upstream from the transcription starting site of PTGR1.
Both variant sequences of PTGR1 with experimentally verified 5' transcripts from the database ElDorado 12-2010 were utilized (Genomatix Gene2promoter software).

b. Preconditioning with 20 μM D3T for 48 h induces hPTGR1 expression in colon cancer cell SW620 and liver cancer cell HepG2 and enhances HMAF cytotoxicity. A typical survival graph is shown above, error bars represent standard deviation. IC₅₀ was calculated from 4 independent experiments by non-linear regression analysis (p < 0.05).

Figure 5 a. Upregulation of PTGR1 by curcumin, resveratrol or D3T. Total RNA (24 h) after treatment at indicated concentrations were analyzed via quantitative RT-PCR. Error bars represent standard error from three independent experiments (p < 0.05).

b. Effects of pretreatment with D3T (20 μM), curcumin (10 μM) or resveratrol (15 μM) on HMAF toxicity in SW620 and HepG2 cells. Error bars represent standard error from three independent experiments (p < 0.05 with the exception of SW620 + 10 μM curcumin).
Tables

Table 1 Kinetic parameters for HMAF metabolism by PTGR1

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmole mg$^{-1}$ min$^{-1}$)</th>
<th>$V_{max}/K_m$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPTGR1</td>
<td>215.1</td>
<td>1.2</td>
<td>5.3</td>
</tr>
<tr>
<td>hPTGR1</td>
<td>4.9</td>
<td>0.6</td>
<td>111.7</td>
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</table>
Table 2 HMAF metabolism and IC₅₀ of HEK293 cells transfected with rPTGR1 vs. hPTGR1

<table>
<thead>
<tr>
<th></th>
<th>Enone reductase activity (nmole/min/mg)</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293-rPTGR1</td>
<td>17.5 ± 7.0</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>HEK293-hPTGR1</td>
<td>247.6 ± 20.4</td>
<td>25 ± 6*</td>
</tr>
</tbody>
</table>

* P < 0.05
Table 3 Effects on HMAF IC$_{50}$ by overexpression of hPTGR1 in HCT15 cells

<table>
<thead>
<tr>
<th>Transfection</th>
<th>hPTGR1 fold change$^a$</th>
<th>HMAF IC$_{50}$</th>
<th>IC$_{50}$ fold change</th>
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</thead>
<tbody>
<tr>
<td>GFP</td>
<td>-</td>
<td>16.9 µM</td>
<td>-</td>
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<tr>
<td>hPTGR1</td>
<td>2</td>
<td>5.7 µM$^*$</td>
<td>3$^*$</td>
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</tbody>
</table>

$^a$Fold change determined by densitometry of immunoblots

* $P < 0.05
Table 4 Pretreatment of HepG2 and SW620 cells sensitizes cells to HMAF

<table>
<thead>
<tr>
<th>precondition</th>
<th>hPTGR1 fold changea</th>
<th>HMAF IC50</th>
<th>IC50 fold change</th>
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</thead>
<tbody>
<tr>
<td>HepG2 Veh</td>
<td>34.2 µM</td>
<td>-</td>
<td></td>
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<tr>
<td>HepG2 D3T</td>
<td>8.9 µM*</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>HepG2 Curcumin</td>
<td>15.3 µM*</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HepG2 Resveratrol</td>
<td>7.4 µM*</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>SW620 Veh</td>
<td>20.1 µM</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SW620 D3T</td>
<td>9.9 µM*</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>SW620 Curcumin</td>
<td>20.3 µM</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SW620 Resveratrol</td>
<td>3.0 µM*</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05 compared to Veh treated population

aFold change determined by densitometry of immunoblots
Scheme 1

1 \( R^1 = \text{OH Illudin S} \)
2 \( R^1 = \text{H Illudin M} \)
3 \( R^2 = \text{CH}_2\text{OH} \)  
   Hydroxymethylacyfluvene (HMAF)
4 \( R^2 = \text{H Acylfluvene (AF)} \)
Figure 1
Figure 2
Figure 3
Figure 4a

Human PTGR1
Variant GXP-227977
Variant GXP-1829305

Nrf2 transcriptional complex — 100 bp  TSS: transcription starting site
Figure 4b
Figure 5a