

Targeting 20-HETE producing enzymes in cancer – rationale, pharmacology, and clinical potential

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Abstract: Studies demonstrate that lipid mediator 20-Hydroxyeicosatetraenoic acid (20-HETE) synthesis and signaling are associated with the growth of cancer cells in vitro and in vivo. Stable 20-HETE agonists promote the proliferation of cancer cells, whereas selective inhibitors of the 20-HETE-producing enzymes of the Cytochrome (CYP450)4A and CYP4F families can block the proliferation of glioblastoma, prostate, renal cell carcinoma, and breast cancer cell lines. A recent observation that the expression of CYP4A/4F genes was markedly elevated in thyroid, breast, colon, and ovarian cancer further highlights the significance of 20-HETE-producing enzymes in the progression of different types of human cancer. These findings provide the rationale for targeting 20-HETE-producing enzymes in human cancers and set the basis for the development of novel therapeutic strategies for anticancer treatment.

Keywords: 20-hydroxyeicosatetraenoic acid, CYP4A, CYP4F, HET0016, eicosanoids

Introduction

The eicosanoid, 20-hydroxyeicosatetraenoic acid (20-HETE), has emerged as a novel signaling molecule contributing to the progression of cancer.^{1–3} Eicosanoids are 20-carbon bioactive lipid mediators generated by enzymatic oxidation of arachidonic acid (AA). These include prostaglandins (products of cyclooxygenases), leukotrienes (products of lipoxygenases), and hydroxyeicosatetraenoic (HETEs) and epoxyeicosatrienoic acids (EETs) (products of cytochrome P450 enzymes).⁴ Even though eicosanoid-mediated modulation of ion transport, renal and pulmonary functions, as well as vascular tone and reactivity have been universally acknowledged,^{5,6} not until recently has it become evident that these lipid mediators are also involved in carcinogenesis.^{7,8} Prostaglandins have subsequently been the most widely and intensely studied group of eicosanoids in cancer biology.⁸ Among prostaglandins, prostaglandin E₂ (PGE₂) has received the most attention as a potential contributor to cancer progression.^{9–11} Indeed, PGE₂ has a potent proliferative effect, is involved in conferring a multidrug resistance phenotype,^{12,13} and it increases tumor growth in ApcMin/+ and azoxymethane mouse models of colorectal cancer.¹⁴ PGE₂ also reversed nonsteroidal anti-inflammatory drug-induced adenoma regression in these mice. Furthermore, inhibition of endogenous PGE₂ resulted in the suppression of intestinal tumorigenesis.¹⁵ These findings are consistent with established PGE₂-mediated signaling, which includes, among others, transactivation of endothelial growth factor (EGF) receptor,^{16–18} and peroxisome proliferator-activated receptor δ .¹⁹ Activation of these signaling cascades resulted in stimulation of cell migration through increased PI3K-Akt signaling in colon cancer cells and increased intestinal epithelial tumor cell survival. Concordantly, PGE₂ has

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also been shown to induce expression of such antiapoptotic proteins as Bcl-2,²⁰ and increase transcriptional activity of a key antiapoptotic regulator, nuclear factor-kappa B (NFκB).²¹ It has also been reported that PGE₂ possesses an angiogenic effect.^{22,23} PGE₂ reversed the antiangiogenic activity of nonsteroidal anti-inflammatory drugs, whereas homozygous deletion of PGE₂ receptor EP2 completely abrogated the induction of vascular endothelial growth factor (VEGF) in APC^{Δ716} mouse polyps.²⁴ This is consistent with earlier studies showing that PGE₂ upregulates VEGF in cultured human fibroblasts,²⁵ and increases VEGF and basic fibroblast growth factor expression through the stimulation of extracellular-signal-regulated kinase (ERK)2/c-Jun N-terminal kinase 1 signaling pathways in endothelial cells.²⁶ Similarly, while not as well studied as PGE₂, PGF_{2α} has been demonstrated to enhance carcinogen-induced transformation of fibroblasts in vitro,⁷ while thromboxane A₂ was reported to promote angiogenesis.²⁷

Compared with prostaglandins, much less is known about the role of lipoxygenases (LOXs) in cancer. Data are accumulating that support the role of 15-LOX-1 as a tumor suppressor, especially in colon cancer.²⁸ On the other hand, overexpression of 12-LOX was strongly associated with poor differentiation and invasiveness of prostate cancer.²⁹ Further, it has been shown that leukotriene B₄ (LTB₄) levels are increased in human colon and prostate cancers,^{30,31} and the expression of LTB₄ receptors is upregulated in human pancreatic cancer.³² Additionally, it has been shown that inhibition of LTB₄ synthesis leads to reduced esophageal adenocarcinoma in a rat model and that blocking the receptor of LTB₄ suppressed the LTB₄-stimulated expression of ERK in colon cancer cells.³³ Other LOX byproducts, such as 12(S) HETE have been reported to mediate the activation of NFκB,³⁴ induce angiogenesis through stimulating VEGF expression in prostate cancer cells,^{35,36} and increase adhesion of B16 murine melanoma cells to endothelial cells via upregulation of αvβ3 integrin expression.³⁷

The role of HETEs and EETs in cancer has been neglected until recently.³⁸ There are mounting data that suggest that products of ω-hydroxylases of the cytochrome P450 (CYP) family of proteins, notably 20-HETE, can play an important role in cell growth and cancer development.³⁸ In this review, we will summarize the findings that provide the rationale for considering 20-HETE producing enzymes as novel targets for anticancer therapy, describe the potential of available pharmacological agents for interfering with 20-HETE synthesis and signaling, and discuss the potential of their clinical application for cancer treatment.

Cellular synthesis of 20-HETE and other eicosanoids

AA is metabolized to eicosanoids through three major pathways: the cyclooxygenase (COX), the LOX, and the CYP-450 monooxygenase pathways, which insert oxygen at different positions in AA to generate the wide variety of lipid mediators. AA metabolized by the COX pathway forms prostaglandins (PGs) and thromboxanes. AA metabolized by LOX pathway produces 15(S), 12(S), 12(R), 8(S), 5(S) HETEs, leukotrienes, and lipoxins. Finally, AA metabolized by the CYP-450 pathway generates 16-, 17-, 18-, 19-, and 20-HETEs and 5-, 6-, 8-, 9-, 11-, 12-, 14-, and 15-EETs.⁴

The COX enzymes (COX1/COX2) catalyze the conversion of AA to an unstable cyclic endoperoxide (prostaglandin H₂, PGH₂), which is further catalytically converted to the various prostanoids including PGD₂, PGE₂, PGF_{2α}, prostacyclin, and thromboxane A₂ via reduction, rearrangement, or isomerization by the terminal synthase enzymes.⁴ It should be noted that 5-, 8-, 12-, and 15-LOX enzymes metabolize AA to several regioisomeric allylic hydroperoxides, which are further reduced into the corresponding leukotrienes and HETEs.⁴ The cytochrome P-450 ω-hydroxylases (CYP4A and CYP4F) metabolize AA to produce HETEs, including 19- and 20-HETE (Figure 1), and cytochrome P-450 epoxygenases (CYP2C and CYP2J) convert AA to the various EETs. To generate 19/20-HETE the ω-hydroxylases of the 4A and 4F gene families of CYP450 introduce an hydroxyl (OH) group at or near the terminal sp³ carbon group of AA.^{4,39,40} The pathways involved in the metabolism of AA are depicted in Figure 2.

Methods of detecting 20-HETE in cells and tissues

Due to the chemical and biological complexity of eicosanoids, the measurement of their concentrations in biological samples must be carefully validated and controlled in the laboratory.

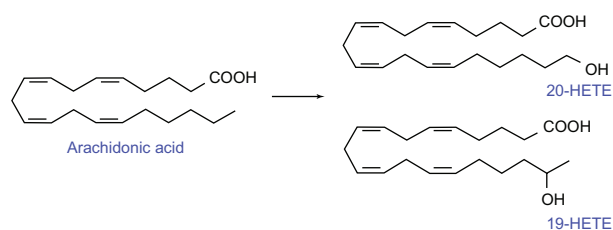


Figure 1 Synthesis of 20-HETE.

Note: ω/ω-1 hydroxylases of CYP4A and CYP4F introduce a hydroxyl group into positions 20 or 19 of arachidonic acid to form 19-HETE and 20-HETE, respectively.
Abbreviations: 20-HETE, 20-hydroxyeicosatetraenoic acid; CYP, cytochrome P450; 19-HETE, 19-hydroxyeicosatetraenoic acid.

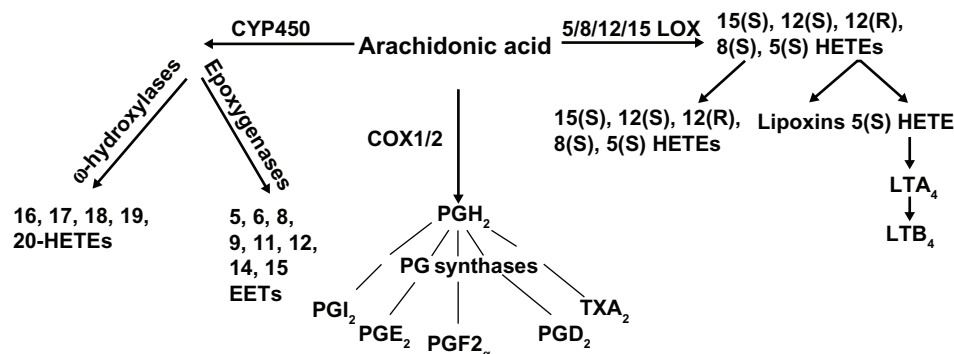


Figure 2 An overview of eicosanoid synthesis pathways.

Note: Schematic overview of three major enzymes of arachidonic acid metabolism and their metabolites.

Abbreviations: CYP, cytochrome P450; LOX, lipoxygenase; EET, epoxyeicosatrienoic acid; PGI₂, prostacyclin synthase; PGE₂, prostaglandin E₂; PGF₂, prostaglandin F₂; PGD₂, prostaglandin D₂; TXA₂, thromboxane A₂; LTA₄, leukotriene A₄; LTB₄, leukotriene B₄; PGH₂, prostaglandin H₂; COX1/2, cyclooxygenase 1/2; HETE, hydroxyeicosatetraenoic acid.

There are several analytical techniques available when it is necessary to determine eicosanoid concentrations in biological samples. In this chapter, we discuss each of these techniques and evaluate the advantages and drawbacks of each methodology.

Radioimmunoassay (RIA) is a commonly used method for the quantitative analysis of eicosanoid levels in biological samples. The assay is based on the competition between the antigen (the eicosanoid being measured) and a constant amount of radioactive homologous antigen for a limited amount of specific eicosanoid antibody. Thus, the necessary reagents include a monospecific antibody, an unlabelled eicosanoid to be used as a standard, and radioactively labeled eicosanoid. The antibody-bound radioactivity is inversely proportional to the concentration of the eicosanoid in the sample. RIA is rapid, sensitive, and relatively inexpensive;^{4,41} however, there are risks associated with using radioactive materials, which accompany all RIA assays.

Similarly, enzyme immunoassay (EIA) is also based on the competition between an eicosanoid and a constant amount of chemiluminescent (eg, acetylcholinesterase or alkaline phosphatase) conjugated eicosanoid for a limited amount of antibodies raised against a particular eicosanoid. The product of the enzymatic reaction is read spectrophotometrically at a certain wavelength after the addition of a corresponding substrate and is inversely proportional to the concentration of the eicosanoid in the samples. EIA kits for 20-HETE measurement (Eagle Biosciences, Nashua, NH, USA; R&D Inc, Detroit, MI, USA; and ALPCO Diagnostics, Salem, NH, USA), as well as kits for other eicosanoids, are commercially available. While EIA is similar to RIA with regard to sensitivity and specificity, the cost of EIA is much higher. Further, both assays are limited by the fact that antibodies to all the known AA metabolites are not readily available.

Another drawback of these assays is nonspecific binding, which results from the cross-reactivity of the antibodies with other eicosanoids of similar structure leading the investigator to overestimate the concentrations of analytes. Further, both of these techniques allow for the analysis of only one analyte at a time, which is time consuming.^{4,41}

Gas chromatography-mass spectrometry (GC-MS) is another technique utilized in the analysis of eicosanoids and is based on the partition of the analyte between the stationary and gaseous phases. This requires molecules that are being analyzed to be volatile and thermally stable at high GC temperatures. To achieve this, polar groups on eicosanoids, which cause tailing during GC-MS, need to be derivatized to more stable trimethylsilyl groups, which is time consuming and complicated.^{4,41}

Liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) is a more practical analysis of eicosanoids. In the analysis of eicosanoids by LC-MS-MS, eicosanoids have to be initially extracted by a solid phase, reverse phase, normal phase (silica), and ion exchange columns. Eicosanoids can also be further extracted using liquid extraction, and depending on the eicosanoid being analyzed, one or both of the extraction methods may need to be utilized. The complex mixture of eicosanoids will then need to be separated by liquid chromatography before being introduced into the source of the mass spectrometer, where ionization takes place allowing for the identification of different eicosanoids. Additionally, the use of stable isotope-labeled internal standards in LC-MS-MS prior to extraction is essential for quantitative analysis of the eicosanoids present in cells, urine, plasma, or tissue homogenates.^{4,41} An example of the detection of eicosanoids by LC-MS-MS is shown in Figure 3. We have used this technique quite routinely for the measurement of 20-HETE in tissue and cellular samples.

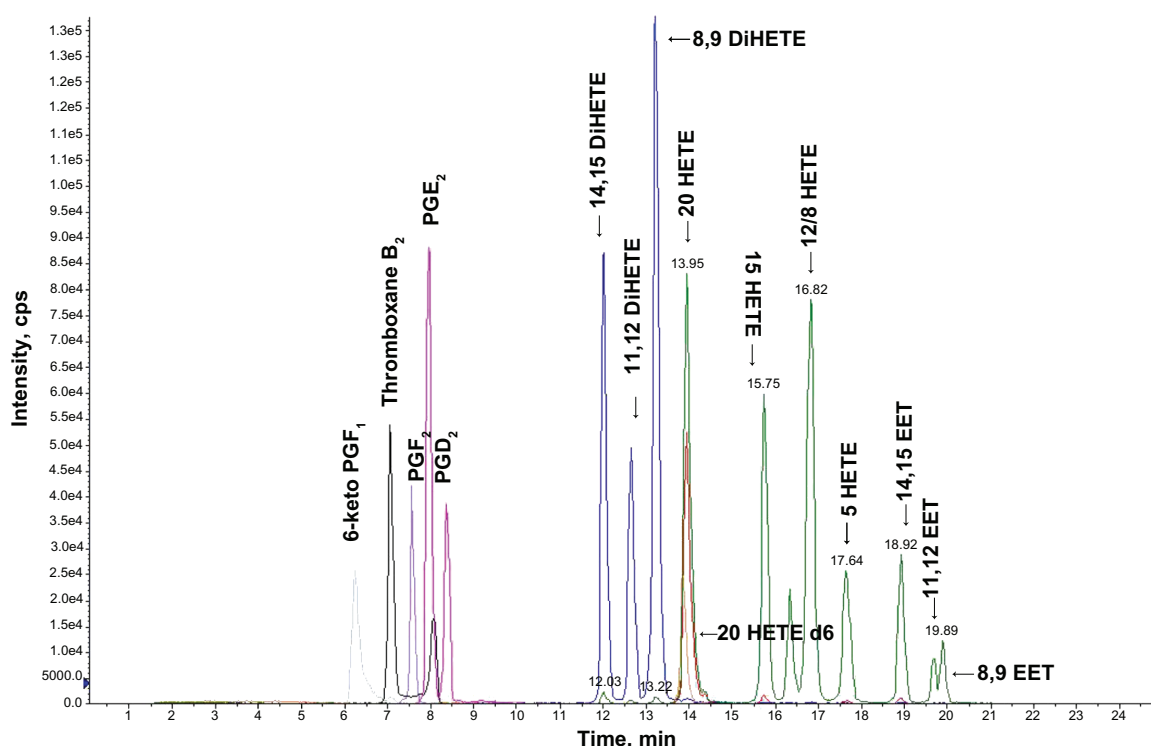


Figure 3 LC-MS-MS detection of HETE standards.

Note: Representative chromatogram generated from LC-MS-MS data, illustrating the separation of various HETEs with different retention times and transitions.

Abbreviations: LC-MS-MS, liquid chromatography-mass spectrometry-mass spectrometry; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; DiHETE, diastereomeric 5,6-dihydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid.

While GC-MS and LC-MS-MS are both sensitive and allow for the analysis of multiple analytes simultaneously, the cost is prohibitive, which limits the usage of this method for 20-HETE detection.

In summary, LC-MS-MS and GC-MS are the most reliable and accurate methods for identifying and quantifying 20-HETE and other eicosanoid levels. However, the high cost of these techniques limits the number of replicates per sample, sometimes leading to inconclusive results. Immunoassays, which are less expensive, recently became an alternative detection method for 20-HETE and other eicosanoids for which antibodies are available. They are more practical, as samples can be run in replicates and a larger amount of samples can be assayed; however, immunoassays can also be problematic due to nonspecific binding. Overall, as each of the detection methods listed above has its drawbacks, it is desirable to combine these assays to accurately measure and confirm levels of 20-HETE and other eicosanoids in biological samples.

20-HETE-producing enzymes

There are four human CYP450 isoforms that catalyze the hydroxylation of AA at the ω - or 20-carbon to produce 20-HETE; they are CYP4A11, CYP4A22, CYP4F2,

and CYP4F3.^{39,40,42} These CYP450 enzymes are encoded by distinct genes and are highly homologous. Analysis of the CYP4A11 gene revealed that, like CYP4A22, it contains 12 exons and eleven introns, and its overall length and exon/intron structure were similar to those of CYP4A22. However, the physical alignment of CYP4A11 and CYP4A22 genes exposed small insertions and deletions in the introns, with the most conserved being intron 8, while introns 3, 6, and 9 showed the highest degree of divergence, resulting in a 96% overall sequence identity between CYP4A11 and CYP4A22 genes. Furthermore, the translated sequences for CYP4A11 and CYP4A22 show that these proteins are of the same length and differ only by 25 residues.⁴³ Similarly, CYP4F2 and CYP4F3 genes share 80% amino acid identity, with highly similar organization of genes.⁴⁴ Of the four 20-HETE-producing enzymes, CYP4F2 is considered the major enzyme involved in the production of 20-HETE in humans accounting for about 70% of 20-HETE production, with an apparent K_M of 23.5 μ M and V_{max} of 7.4 min^{-1} while CYP4A11 has a tenfold higher K_M of 228.2 μ M and V_{max} of 49.1 min^{-1} . Besides kinetic studies, the role of CYP4F2 as the predominant AA ω -hydroxylase in the production of 20-HETE, has been further confirmed by immunoinhibition studies, which showed that antibodies to CYP4F2 elicited

marked inhibition (93%) of 20-HETE formation, whereas antibodies to CYP4A11 only elicited 13% inhibition.⁴² It was recently reported that overexpression of a novel CYP4 family member, CYP4Z1, was associated with increased levels of 20-HETE in breast cancer cells.⁴⁵

The abundance of 20-HETE-producing enzymes in mammals makes it hard to use the knockout approach to study their relevance for cancer progression.³⁹ In mice, the two enzymes primarily responsible for 20-HETE production are CYP4A10 and CYP4A12, with CYP4A12 being the predominant 20-HETE synthase in the mouse kidney.⁴⁶ In rats, there are four isoforms of 20-HETE-producing enzymes: CYP4A1, CYP4A2, CYP4A3, and CYP4A8. Among them, CYP4A1 has the highest catalytic activity toward AA ω -hydroxylation. It is followed by CYP4A2 and CYP4A3, which display similar activity. CYP4A8 also contributed to 20-HETE formation in rat kidney.⁴⁷

Disruption of the mouse CYP4A10 gene caused hypertension, which was dietary salt sensitive.⁴⁸ CYP4A12 knockout mice were not described. Recently, rat knockouts of CYP4A2 and CYP4A3 were generated using a zinc finger nuclease approach as a part of the PhysGen Knockout program (“Mechanistic Characterization of Genes for Hypertension and Renal Disease”) at the Medical College of Wisconsin (Milwaukee, WI, USA). This program aimed to knock out a large number of genes, nominated by genome-wide association studies as relevant for the development of hypertension and renal disease, and the generated rat strains are available for distribution. So far, neither mouse nor rat knockouts of 20-HETE-producing enzymes have been utilized in cancer-related studies.

20-HETE actions and signaling

It should be noted that 20-HETE, being a potent vasoconstrictor, plays a complex role in the regulation of blood pressure and vascular tone. The vasoconstriction activity of 20-HETE in rat aortic rings was among the first 20-HETE actions described.⁴⁹ Specifically, 20-HETE mediates autoregulation of cerebral blood flow.⁵⁰ Renal actions of 20-HETE are linked to the regulation of Na⁺ transport and the pressure-natriuresis response.^{51,52} Importantly, 20-HETE inhibits Na⁺ transport in renal proximal tubules,⁵³ but not in cortical collecting ducts, where inhibition is likely to be mediated by EETs.^{54,55} Inhibitors of 20-HETE formation promote salt-sensitive hypertension and renal injury in the rat model.⁵⁶ For an excellent summary of the renal and vascular actions of 20-HETE and the role of 20-HETE in the control of hypertension, see the review by Williams et al.⁵² The role of

20-HETE in physiological and pathological vascularization was also recently reviewed.⁵⁷

In addition to vasoactive effects, 20-HETE stimulates mitogenic and angiogenic responses both *in vitro* and *in vivo*.³⁹ Both mitogenic and angiogenic activities are important for the ability of 20-HETE to support the proliferation of cancer cells and tumor growth; 20-HETE has been shown to induce cell proliferation and DNA synthesis in vascular smooth muscle and proximal tubule cell cultures, as well as in a number of cancer cell lines. The proangiogenic activity of 20-HETE with regard to endothelial cell population is through the promotion of proliferation, migration, and cell survival. These 20-HETE actions were accompanied by increased VEGF expression and release.^{1,58} VEGF is the major driver of tumor angiogenesis.⁵⁹ These studies support the idea that 20-HETE signaling contributed to cell growth, invasion, and angiogenesis in the regulation of tumor development.

The biggest challenge with attempts to define 20-HETE signaling is that to date, the specific cellular receptor of 20-HETE has not been identified. In striking contrast to PGs (whose G-protein coupled receptors are well studied and characterized), no reliable information about the nature of HETE receptors in general and the 20-HETE receptor in particular, is available. Correspondingly, specific 20-HETE antagonists preventing the binding of 20-HETE to its receptor are missing from the arsenal of tools for the treatment of 20-HETE-mediated pathologies, a circumstance which continues to delay the development of 20-HETE-focused therapeutic strategies. The situation was improved with the introduction of specific inhibitors of 20-HETE synthesis and antagonists of 20-HETE signaling, which will be discussed below. It also must be taken into consideration that enzymes that synthesize 20-HETE are microsomal enzymes, and 20-HETE, when released intracellularly, could act as an intracellular second messenger and interact directly with intracellular targets. Therefore, the possibility that 20-HETE and other HETEs act without binding to canonical transmembrane receptors cannot be ruled out, even though it would make them different from other eicosanoids.

In the absence of an identified 20-HETE receptor, studies of 20-HETE signaling are limited to description of cellular signaling pathways being activated following addition of 20-HETE to cultured cells and administration of 20-HETE to animals. Multiple signaling cascades were reported to be activated by 20-HETE in a variety of cell systems.^{57,60,61} In addition, 20-HETE induced activation of Raf/MEK/ERK, signaling a cascade secondary to the activation of c-Src and EGF receptor in renal epithelial cells.⁶² Moreover, 20-HETE triggers NF κ B and ERK activation in

human endothelial cells.⁶⁰ Furthermore, 20-HETE can also activate the small-GTPase Ras, which plays a critical role in the regulation of cell growth;⁶³ 20-HETE was reported to signal through the cyclic adenosine monophosphate/protein kinase A (PKA)-phosphorylase kinase-glycogen phosphorylase pathway to induce hyperglycemia.⁶⁴ NAHPH-oxidase-derived superoxide mediated 20-HETE-induced activation of L-type Ca^{2+} channels via a PKC-dependent mechanism in cardiomyocytes.⁶¹ A diagram showing the various 20-HETE-mediated signaling pathways and their (probable) interactions is included (Figure 4).

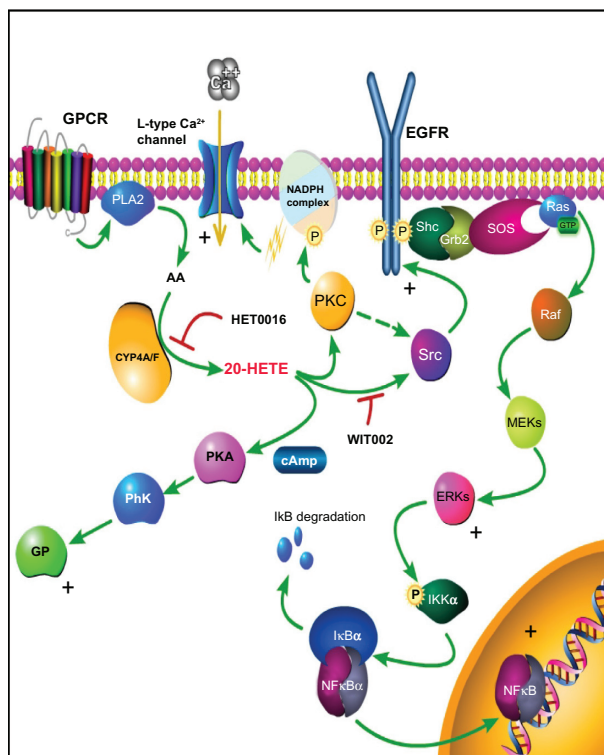


Figure 4 Proposed mechanism of action of 20-HETE.

Notes: Mitogens and ligands of G-protein-coupled receptors activate PLA2 and induce release of arachidonic acid, which is converted by CYP4A/F to 20-HETE. Then, 20-HETE, acting presumably via Src, promotes tyrosine phosphorylation of EGFR and consequently activates the Ras-Raf-MEK-ERK cascade, which leads to the proliferation of cancer cells. One of cytoplasmic targets of ERK is I-kappaB kinase-alpha, which phosphorylates I-kappaB-alpha, resulting in the degradation of I-kappaB-alpha by the proteasome and the translocation of NF-kappaB to the nucleus, where it positively regulates the transcription of a number of genes involved in immune and inflammatory responses including VEGF. In addition, 20-HETE also activates cAMP-dependent PKA-phosphorylase kinase-GP (glycogen phosphorylase pathway) to induce hyperglycemia. Besides, 20-HETE stimulates NADPH oxidase-derived superoxide production, which activates L-type Ca^{2+} channels via a PKC-dependent mechanism. Inhibitory interactions are shown by red lines with bars. Stimulatory interactions are shown by green lines with arrows. Activation of the principal signaling cascade is shown by +.

Abbreviations: 20-HETE, 20-hydroxyeicosatetraenoic acid; GPCR, G protein-coupled receptor; EGFR, endothelial growth factor; PLA2, phospholipase A2; NADPH, nicotinamide adenine dinucleotide phosphate; SOS, salt overly sensitive; AA, arachidonic acid; CYP, cytochrome P450; PKC, protein kinase C; PKA, protein kinase A; PhK, phosphorylase kinase; GP, glycogen phosphorylase pathway; cAMP, cyclic adenosine monophosphate; MEK, MAP kinase kinase; ERK, extracellular signal-regulated kinases; I-kappaB, I-kappaB; NF-kappaB, nuclear factor-kappaB; VEGF, vascular endothelial growth factor; HET0016, N-Hydroxy-N'-(4-butyl-2-methylphenyl) formamidate; WIT002, 20-hydroxyeicos-6(Z),15(Z)-dienoic acid.

Expression of 20-HETE-producing enzymes in human cancers

While 20-HETE induces mitogenic and angiogenic responses in cancer cells and inhibitors of the 20-HETE pathway have been shown to reduce the growth of brain and kidney tumors at least in animal models,^{65,66} the expression pattern of 20-HETE-producing enzymes in human cancers has not been addressed. We have demonstrated that the 20-HETE-producing enzymes CYP4A/F are upregulated in human cancer tissue samples.⁶³ In this study, TissueScan cancer survey panels obtained from OriGene (OriGene Technologies, Inc, Rockville, MD, USA) containing prenormalized cDNA from multiple cancer tissues were used to run real-time polymerase chain reaction (Sybr Green[®], Life Technologies, Carlsbad, CA) to analyze the expression of CYP4A and CYP4F genes at the messenger ribonucleic acid (mRNA) level. The tissue samples used for each type of TissueScan panel were obtained from independent patients, covered various disease stages, and matched control normal tissues, and these samples are included for comparison between normal and disease samples. Analysis of 20-HETE-producing CYP enzymes in various human cancer tissues demonstrated that CYP4A/F enzymes were upregulated at the mRNA level in human thyroid, breast, colon, and ovary cancers when compared to corresponding normal tissue.⁶³ Analysis of human lung cancer tissue samples also revealed increased levels of CYP4F/A enzymes; even though a dramatic increase of expression of 4F2 and 4F3 were detected only in one of nine cancer tissue samples, two other cancer samples (indicated as E8 and E10) demonstrated increased expression of 4F2, which was not detectable in matched control normal tissues (Figure 5). Most CYP450 enzymes are expressed in the liver and are involved in the metabolism of drugs and foreign chemicals. Both normal and cancerous liver cells are characterized by the increased expression of 20-HETE-producing enzymes. The prostate is also rich with AA-metabolizing enzymes. Any increase in the expression of 20-HETE-producing enzymes in the liver and prostate will be more difficult to detect because it will be masked by substantial expression in normal cells; this is not the case with other tissues. Primers used in real-time polymerase chain reaction experiments to detect the expression of CYP4A/F genes at the mRNA level are listed in Table 1. Using Western blot analysis of an OncoPair INSTA-Blot[™] PVDF membrane (IMGEX Corporation, San Diego, CA, USA) containing 14 lanes of alternating ovarian tumor and matched normal adjacent tissue lysates from seven patient donors (IMGEX Corporation) and immunohistochemistry analysis, we further

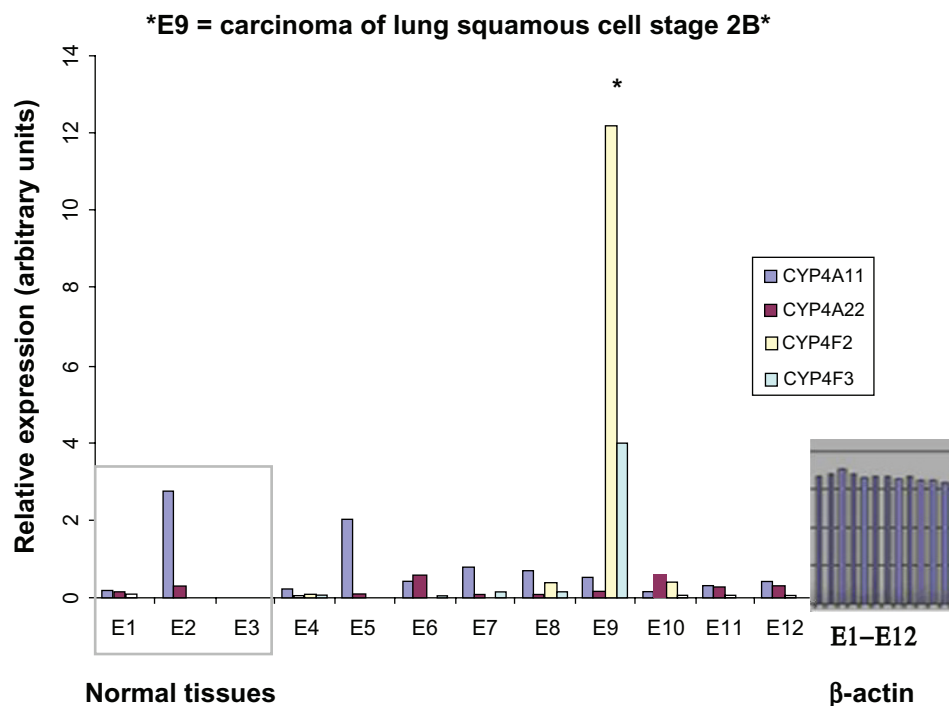


Figure 5 Expression of CYP4A and CYP4F genes at the mRNA level is elevated in human lung cancer tissue samples.

Notes: Real time-PCR of lung TissueScan cancer survey panel using primers for CYP4A/F genes. Matched control normal tissues are included for comparison between normal and disease samples. The amount of PCR product is correlated with the amount of original RNA transcript and the data are expressed in arbitrary units. *Indicates tumor samples demonstrating striking up-regulation in CYP4A/F enzymes.

Abbreviations: CYP, cytochrome P450; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; RNA, ribonucleic acid.

demonstrated that the expression of CYP4F2 (the major 20-HETE-producing enzyme) was increased at the protein level in ovarian cancer when compared to normal adjacent tissue.⁶³

Agonists, antagonists, and inhibitors of the synthesis of 20-HETE

A hydroxyphenylformamidine derivative, HET0016 [N-hydroxy-N'-(4-butyl-2-methylphenyl)-formamidine], was shown to be a potent and selective inhibitor of 20-HETE synthesis in rat and human renal microsomes.⁶⁷ To retain high inhibitory activity, N-hydroxyformamidine had to be unsubstituted, with the substituent at the paraposition of N-hydroxyformamidine decreasing the inhibitory activity most drastically.⁶⁸

In the absence of antagonists preventing the binding of 20-HETE to its probable transmembrane receptor, 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (WIT002) has emerged as an antagonist of 20-HETE signaling (Figure 6).⁶⁹ WIT002 is a noncyclooxygenase metabolizable stable analog of 20-HETE, which antagonizes the vasoconstrictor effects of 20-HETE in vitro and in vivo.^{69,70} WIT002 also inhibits the stimulatory effect of 20-HETE upon the platelet-derived growth factor-mediated migration of vascular smooth muscle cells.⁷¹ N-[20-hydroxyeicosa-6(Z),15(Z)-dienoyl]glycine (6,15-20-HEDGE) is an analog of WIT002 that may be more efficacious because of the addition of a glycine group, which may help the compound enter cells or prolong its action by protecting it from β -oxidation. In addition, synthetic stable analogs of

Table I Primers used to detect human genes encoding CYP4A/F by real-time PCR

Enzyme	Forward	Reverse
CYP4A11	5'-ATGAAGTGTGCCTTCAGCCA-3'	5'-AAGGCATTCCTCACACGGG-3'
CYP4A22	5'-AATGGGAAGAGCTCCTTGGC-3'	5'-AAGGCATTCCTCATACAGC-3'
CYP4F2	5'-AAGCACCCAGAATACCAGGA-3'	5'-TCATGCACATGGTCAGGAAG-3'
CYP4F3	5'-CTGTGCGGCAGGAGGTACAAG-3'	5'-CCTCAGGCTCTCCTTAATGC-3'

Note: The 20-HETE-producing CYP450 isoform-specific primer sequences to detect the expression of CYP4A and CYP4F genes at the mRNA level in human cancer tissues are outlined.

Abbreviations: CYP, cytochrome P450; PCR, polymerase chain reaction; 20-HETE, 20-hydroxyeicosatetraenoic acid; mRNA, messenger ribonucleic acid.

20-HETE N-[20-hydroxyeicosa-5(Z),14(Z)-dienoyl]glycine (5,14-HEDGE) and 20-hydroxyeicosa-5(Z), 14(Z)-dienoic acid (5,14-20-HEDE) (also termed WIT003) were shown to prevent the impairment of cardiorenal functions suggesting a therapeutic potential for 20-HETE analogs.^{72,73}

The question of the specificity of inhibitors of 20-HETE synthesis and signaling is a particularly important one. The ability of HET0016 to specifically inhibit the formation of 20-HETE in vivo has been confirmed using LC-MS-MS profiling of all the COX, LOX, and CYP450 eicosanoids in a variety of tissues following in vitro and in vivo administration.⁷⁴ The data supporting the specificity of the agonist WIT003 and the antagonist 6,15-20-HEDGE is less direct.⁶⁹

20-HETE in cancer cell proliferation and tumor growth

Despite multiple reports of the anticancer activity of inhibitors of 20-HETE synthesis and signaling, there is a shortage of data that demonstrate increased levels of 20-HETE in

human cancers. One example is provided by prostate cancer patients, who were shown to have urinary levels of free 20-HETE that were significantly higher than those of normal men, even though a correlation between the concentration of 20-HETE and grade of tumor was not found.⁷⁵

The human glioblastoma cell line, U251, is a malignant glioma cell model, which recapitulates the most prominent histological and immunohistochemical features of human glioblastoma multiforme, a highly aggressive brain tumor with a very poor prognosis.⁷⁶ HET0016 decreased both basal and EGF-induced U251 cell proliferation, an effect that was nullified by the addition of the 20-HETE agonist, WIT003.⁷⁷ HET0016 also reduced angiogenic response to U251 cells measured by a cornea pocket angiogenesis assay.⁷⁸ The 9L gliosarcoma cell line has been extensively used to study chemotherapy and radiation effects upon growth of glioma cells, even though the 9L glioma model is characterized by significant differences from human glioblastoma multiforme.⁷⁶ HET0016 efficiently inhibited 9L cell proliferation, induced apoptosis in these cells, and

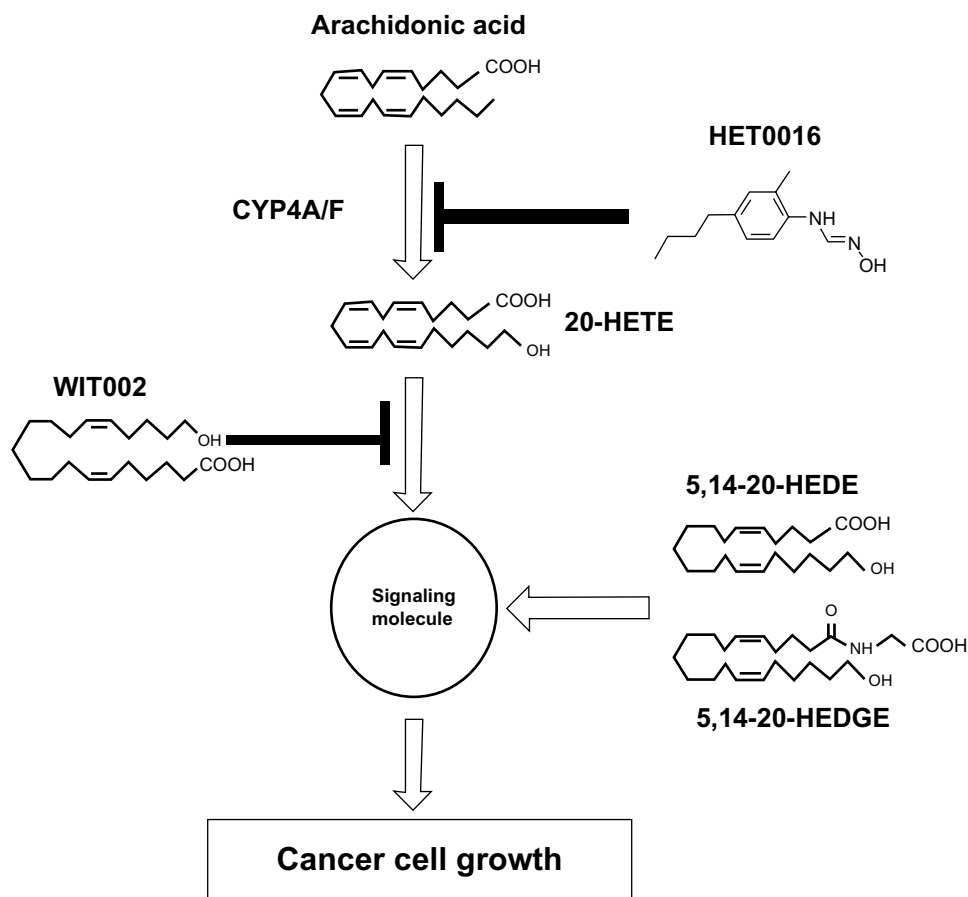


Figure 6 Inhibitors, agonists, and antagonists of 20-HETE.

Note: Depicted are the chemical structures of inhibitors of 20-HETE producing ω -hydroxylases as well as 20-HETE analogs that exhibit agonist or antagonist activity.

Abbreviations: 20-HETE, 20-hydroxyeicosatetraenoic acid; 5,14-20-HEDGE, N-[20-hydroxyeicosa-5(Z), 14(Z)-dienoyl]glycine; 5,14-20-HEDE, 20-hydroxyeicosa-5(Z), 14(Z)-dienoic acid. CYP450, Cytochrome P450; WIT002-20-hydroxyeicosa-6(Z),15(Z)-dienoic acid; HET0016, N-Hydroxy-N'-(4-butyl-2-methylphenyl) formamidine.

prevented the proliferative effect of platelet-derived growth factor.⁶⁵ Furthermore, HET0016 also prevented the growth of 9L cells *in vivo*, when 9L cells were implanted into the forebrain of Fisher rats. In the absence of HET0016, control animals implanted with 9L cells display rapidly growing tumor, whereas rats treated with HET0016 exhibited much smaller tumors with a lesser degree of vascularization.⁶⁵ Nevertheless, careful consideration should be implemented when interpreting HET0016 anticancer actions in U251 and 9L cells, because both U251 cells and 9L cells failed to synthesize 20-HETE in the presence of AA.⁷⁷ One possible explanation is that 20-HETE synthesized in these cells is rapidly metabolized either by β -oxidation or by COX, and therefore escapes detection by LC-MS-MS.³⁹ The other possibility is that mechanisms of HET0016 action are independent of the inhibition of 20-HETE synthesis in U251 and 9L cells. However, the enforced expression of CYP4A1 in U251 cells caused an increased cell growth both *in vitro* and *in vivo*, strongly supporting the significant role of 20-HETE in the progression of glioblastomas.⁷⁹

Non-small-cell lung cancer is among the leading causes of cancer death in the world; 20-HETE generated by CYP4A11 promoted lung cancer angiogenesis and metastasis by upregulation of VEGF and Matrix metalloproteinase 9 (MMP-9) via PI3 kinase and ERK1/2 signaling in human non-small cell lung cancer cells.⁸⁰ In addition, 20-HETE signaling was also shown to play a role in tumor angiogenesis and growth in human breast cancer.⁴⁵ Analysis of mRNA levels encoding 20-HETE-producing CYP enzymes revealed increased levels of CYP 4F2 in some human lung cancer tissue samples (Figure 5).

With an estimated 64,770 new cases of cancers arising from the kidney and renal pelvis in the USA in 2012, renal cell carcinoma is among the leading causes of cancer death. Renal cancer is notoriously resistant to conventional therapies (chemotherapy, hormonal therapy, and radiotherapy), and metastatic forms of clear-cell renal carcinoma are usually associated with very poor patient survival rates.⁸¹ Thus, the lack of an effective therapy demands the urgent investigation and development of any novel treatment strategy. Notably, HET0016 and WIT002 inhibit the proliferation of two different human renal cell carcinoma cell lines, 786-O and 769-P, *in vitro*, whereas the proliferation of primary normal human proximal tubule epithelial cells was not affected by these drugs.⁶⁶ Both the TNF-related apoptosis-inducing ligand (TRAIL)-sensitive renal cell carcinoma line 769-P and the TRAIL-resistant renal cell carcinoma line 786-O were equally sensitive to the antiproliferative effects of HET0016.

These selective antiproliferative effects of HET0016 and WIT002 on renal cancer cells are of particular interest, since renal cell carcinomas are highly refractory to conventional chemotherapy and even radiation therapy. WIT002 was able to decrease the growth of tumors arising from human kidney cancer epithelial cells *in vivo*.⁶⁶ These data were the first to indicate the ability of this class of agents to suppress the growth of tumors of human origin.

Overall, 20-HETE plays an important role in the proliferation of prostate cancer cells, both androgen-responsive LNCaP and androgen-nonresponsive PC3 (personal communication from R Roman, Jackson, MS, USA).⁸² CYP4F2/3 might be the source of androgen-driven 20-HETE synthesis via androgen receptor-dependent pathways.

In summary, the addition of inhibitors of 20-HETE synthesis and signaling significantly inhibited the growth of cancer cells both *in vitro* and sometimes *in vivo*. Such results have been obtained using human prostate cancer cells, MDA-MB 231 breast cancer cells (personal communication from R Roman, Jackson, MS, USA), U235, 9L and RG3 glioblastoma cell lines, as well as 780P and 769P renal epithelial cancer cell lines. Together with data demonstrating an increased level of mRNA encoding enzymes responsible for 20-HETE generation, these results provide a compelling reason to study the role of 20-HETE in promoting the proliferation of several forms of human cancer.

Clinical potential of targeting 20-HETE-producing enzymes

Despite dramatic advancements in the treatment of some types of cancer, effective clinical therapies for common human cancers such as lung, ovarian, and renal cancers remain limited. An involvement of AA pathways in the proliferation of different forms of cancer is well documented and there are multiple studies addressing the role of cyclooxygenases and LOX in particular types of cancer.^{83–87} These results have raised enthusiasm for the use of COX-2 inhibitors for anticancer therapy, but the risk of cardiovascular thrombotic events associated with the usage of selective COX-2 inhibitors is a significant concern.^{88,89} Recent studies that have demonstrated the antiproliferative effect of inhibitors of 20-HETE synthesis and the overexpression of CYP4A/F enzymes in human cancers (Table 2) suggest that targeting enzymes responsible for the generation of 20-HETE could be an alternative therapeutic approach for cancer treatment. So far, the role of the products of cytochrome P450 enzymes in control of cancer is insufficiently studied,⁸⁷ and there

Table 2 Expression of CYP4A and CYP4F in human cancer samples and the effects of inhibitors of 20-HETE synthesis and signaling upon cancer cells of different origin

Human cancer type	Estimated new cases in the United States in 2012	Estimated deaths in the United States in 2012	Increased expression of CYP4A or CYP4F enzymes in cancer tissues	Antiproliferative effect of HET0016	Antiproliferative effect of WIT002	References
Breast cancer	226,870 (female) 2190 (male)	39,510 (female) 410 (male)	4F2, 4A11, CYP4Z1	Yes	Not studied	63,45
Colon and rectal cancer	103,170 (colon) 40,290 (rectal)	51,690 (colon and rectal combined)	4F2, 4A22, 4F3	Not studied	Not studied	63
Ovarian cancer	22,280	15,500	4A11, 4A22, 4F2	Not studied	Not studied	63
Kidney cancer	64,770	13,570		Yes	Yes	66
Lung cancer	226,160	160,340	4F2	Yes	Yes	63,80
Prostate cancer	241,740	28,170	Both normal and cancer tissues have high levels of expression	Yes	Not studied	63,82
Thyroid cancer	56,460	1780	4A11, 4A22, 4F2,	Not studied	Not studied	63
Liver cancer	28,720	20,550	Both normal and cancer tissues have high levels of expression	Not studied	Not studied	63
Brain tumor	22,910	13,700		Yes	Yes	65,77,79

Abbreviations: CYP, cytochrome P450; 20-HETE, 20-hydroxyeicosatetraenoic acid; HET0016, N-Hydroxy-N'-(4-butyl-2-methylphenyl) formamidine; WIT002, 20-hydroxyeicos-6(Z),15(Z)-dienoic acid

are no cancer-related clinical trials focusing on 20-HETE synthesis and signaling. The tumor histological type could influence the pharmaceutical agents' capacity to decrease 20-HETE production or signaling.⁸⁰ It is necessary to confirm the mechanism of HET0016 action and identify molecules that are the direct targets of 20-HETE in cancer cells. Furthermore, even though an overexpression of CYP4A/F enzymes has been shown in different types of human cancer (Table 1), studies using a larger number of human tumor samples will be needed to ascertain that the upregulation of CYP4A/F expression occurs in different types of human cancers. These deficiencies notwithstanding, the potential of targeting 20-HETE-producing enzymes to develop into a novel therapeutic strategy for anticancer treatment is high.

Summary

The CYP4A and CYP4F families of cytochrome P450 enzymes catalyze the omega-hydroxylation of AA to form the lipid mediator, 20-HETE.^{90,91} Furthermore, 20-HETE stimulates mitogenic and angiogenic responses in vitro and in vivo;⁹² it also contributes to cancer cell proliferation and tumor growth.⁶⁵ The stable 20-HETE agonists promote the proliferation of cancer cells, whereas selective inhibitors of the CYP4A and CYP4F families prevent the proliferation of glioblastoma, prostate, renal adenocarcinoma, and breast cancer cell lines. mRNAs encoding CYP4A/4F enzymes are markedly elevated in thyroid, breast, colon, and ovarian cancer. These findings provide the rationale for targeting 20-HETE-producing enzymes in human cancers and could be

the basis for the development of novel therapeutic strategies for anticancer treatment.

Acknowledgments

Supported by following grants: Johnson & Johnson grant; Wisconsin Breast Cancer Showhouse Research Grant; NIH grant R21DK088018; Fraternal Order of Eagles Research Grant; and a grant from the Advancing a Healthier Wisconsin Program and The Medical College of Wisconsin Cancer Center.

Disclosure

The authors report no conflicts of interest in this work.

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