There and Back Again: A Perspective on 20 Years of CYP4Z1

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Running Title
20 Years of CYP4Z1

Abbreviations
14,15-dihydroxy eicosatrienoic acid (14,15-DiHET); 1-aminobenzotriazole (ABT); 1-benzylimidazole (1BI); 20-hydroxyeicosatetraenoic acid (20-HETE); 3-((4-(1H-imidazol-1-yl)benzamido)methyl)benzoic acid (inhibitor 9); 8-[(1H-benzotriazol-1-yl)amino]octanoic acid (8-BOA); arachidonic acid (AA); competing endogenous RNA (ceRNA); cytochrome P450 (CYP); cytochrome P450 reductase (CPR); epidermal growth factor (EGF); epoxyeicosatrienoic acid (EET); estrogen receptor alpha (ERα); ethyl (E)-4-(4-(N-hydroxyformimidamido)phenyl)butanoate (7c); fibroblast growth factor (FGF); gas chromatography-mass spectrometry (GC-MS); lauric acid (LA); liquid chromatography with tandem mass spectrometry (LC-MS/MS); luciferin-4F12 (Luc-4F12); luciferin-benzyl ether (Luc-BE); mechanism-based inactivator (MBI); mitogen-activated protein kinase (MAPK); myristic acid (MA); 4-hydroxy-N'-(butyl-2-methylphenyl)-formamidine (HET0016); nickel nitrilotriacetic (Ni-NTA); phosphoinositide 3-kinase/protein kinase B (PI3K/Akt); platelet-derived growth factor (PDGF); polyunsaturated fatty acid (PUFA); soluble epoxide hydrolase (sEH); time-dependent inhibition (TDI); triple negative negative breast cancer (TNBC); vascular endothelial growth factor (VEGF)

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Abstract

Cytochrome P450 4Z1 (CYP4Z1), a highly expressed CYP gene in breast cancer, was one of the last CYPs to be identified in the human genome, some twenty years ago. CYP4 enzymes typically catalyze ω-hydroxylation and metabolize ω3 and ω6 polyunsaturated fatty acids (PUFAs) to bioactive lipid metabolites that can influence tumor growth and metastasis. These attributes of CYP4Z1 make it an attractive target for new chemotherapeutic drug design, as a potential biomarker for selection of patients that might respond favorably to drugs and for developing enzyme inhibitors as potential therapeutic agents. This review summarizes the current state of knowledge regarding the advancing biochemistry of CYP4Z1, its role in breast cancer and the recent synthesis of selective chemical inhibitors of the enzyme. We identify gaps that need to be filled to further advance this field and present new experimental data on recombinant CYP4Z1 expression and purification of the active catalytic form.

Significance Statement

In breast cancer, an unmet need is the availability of highly effective therapeutic agents, especially for triple negative breast cancer. The relevance of the work summarized in this mini-review is that it identifies a new potential drug target, CYP4Z1, and discusses ways in which the gene product’s catalytic activity might be modulated in order to combat this malignancy and limit its spread.

Historical Perspective

The initial identification of CYP4Z1 occurred in 2004 following screening of a transcriptome database for expressed sequences that were upregulated in mammary gland and breast carcinoma (Rieger et al., 2004). These workers then cloned a cDNA corresponding to a new 505 amino acid CYP from breast carcinoma cell line SK-BR-3 and used RT-PCR to demonstrate the mammary-restricted (Table 1) nature of CYP4Z1 mRNA that showed overexpression in ~50% of breast cancer samples analyzed (Radvanyi et al., 2005).

CYP4Z1 is one of 13 human CYP4 enzymes (Table 1). Many of the CYP4 family members, including CYP4Z1, are ‘orphan’ P450s in that their substrate selectivity’s and/or endogenous substrates are unknown. CYP4 enzymes typically catalyze ω-hydroxylation reactions with both xenobiotics and endogenous compounds, and so their catalytic capabilities are critical to human health and disease. An unusual structural feature of many CYP4 enzymes is the presence of a covalently linked heme in ~2/3 of the members of this CYP family (Ortiz de Montellano, 2008). This is an autocatalytic event involving an ester bond forged between a Glu residue in the I-helix and the C-5 methyl of the heme group, as has been confirmed by biochemical analysis and the crystal structure of CYP4B1 (Hsu et al., 2017). CYP4Z1 possesses an Ala residue, not a Glu, at the requisite I-helix position and so would not be expected to have this covalent link (Table 1). However, experimental verification has yet to be obtained. This is one of the gaps in the basic biochemistry of CYP4Z1 that can be addressed by higher yield purification of the enzyme.

The first functional study involving CYP4Z1 came from Prof. Bureik’s laboratory in 2009. This group successfully expressed the human enzyme in yeast that had been co-expressed with the required electron transfer protein, cytochrome P450 reductase (CPR), thereby enabling whole cell biotransformation studies. After incubation with lauric (LA) and myristic acids (MA), gas chromatography-mass spectrometry (GC-MS)
analysis of derivatized extracts revealed the formation of four monohydroxylated metabolites, none of which were \( \omega \)-hydroxylated products. Instead, unique patterns of the \( \omega \)-2, \( \omega \)-3, \( \omega \)-4 and \( \omega \)-5 metabolites were detected with LA generating predominantly the 8-hydroxy (\( \omega \)-4) product and myristic acid mainly forming the 12-hydroxy (\( \omega \)-2) metabolite (Zollner et al., 2009).

These findings bolstered the idea that it might be possible to develop a CYP4Z1-dependent prodrug activation strategy to combat breast cancer, which had first been suggested by Rieger et al. as early as 2004. Prevention of cancer metastasis by chemical inhibitors could possibly be another therapeutic strategy in breast cancer if CYP4Z1 generates a pro-angiogenic metabolite, like 20-hydroxyeicosatetraenoic acid (20-HETE), that is formed from arachidonic acid (AA) by several CYP4 enzymes (Alexanian & Sorokin, 2013). Consequently, this mini-review focuses primarily on the emerging biochemistry of CYP4Z1 with an emphasis on its substrate selectivity and related development of chemical inhibitors, as they might be applied to treat breast cancer.

### CYP4Z1 and Breast Cancer

Cancer is a leading cause of death worldwide, with an estimated 10 million cancer deaths per year (World Health Organization, 2022). On a more positive note, in the US and other western countries, the incidence rates appear to be plateauing and breast cancer mortality has been dropping since the 1990s to a rate of \( ~15 \) per 100,000 in 2012 (Torre et al., 2015). These improvements are likely a combination of earlier pre-screening and improved treatments. However, much remains to be done, especially for cases of triple negative breast cancer (TNBC), which accounts for 10-20% of all cases and is refractory to treatment with endocrine therapy (Kumar & Aggarawal, 2016.)

CYP enzymes play important roles in cancer through modulation of chemotherapy, metastases and carcinogenesis itself (Alzahrani and Rajendran, 2020). While CYP activity is typically reduced in cancer due to an increase in inflammatory cytokines that decrease transcription of the major drug-metabolizing enzyme, CYP3A4, in the liver (Harvey & Morgan, 2014), several CYP4 enzymes are upregulated in cancer states (Alexanian & Sorokin, 2013). Interrogation of the Human Protein Atlas for CYP4Z1 expression in normal and cancer tissues confirms that CYP4Z1 is preferentially localized to the breast and reveals the enzyme’s dramatic increase in breast cancer (Figure 1 A, B). Consequently, modulation of CYP4Z1 activity has attracted attention in breast cancer, perhaps even serving as a ‘silver bullet’ for treating this disease (Yang et al., 2017).

Strong evidence has been put forward that CYP4Z1 promotes angiogenesis and tumor growth when over-expressed in T47D cells (Yu et al., 2012). More recent analysis of clinical samples from patients with TNBC demonstrated a high incidence of CYP4Z1 expression in patients with advanced grades, later stages and larger tumor sizes (Yousef M Al-Saraireh, 2021\(^a\)). Similar studies in ovarian, colon, cervical, and bladder cancers reinforced the conclusion that over-expression of CYP4Z1 and poor prognosis are common features of these malignancies (Yousef M Al-Saraireh, 2021\(^b,c,d\); 2022). This prompted these authors to suggest that CYP4Z1 could be a biomarker and a potential drug target for these solid cancers. CYP4Z1 autoantibody titers have been reported to be high in the sera of breast cancer patients but absent in the sera of healthy patients (Nunna et al., 2017). Follow-up studies indicated that, while CYP4Z1 autoantibodies could also be detected in ovarian, prostate and colon cancers, their diagnostic value as a biomarker for cancer progression appeared limited, but that larger patient sample sizes needed to be evaluated (Khayeka-Wandabwa et al., 2022\(^a\)).

Delineation of a metabolically-based role for CYP4Z1 in breast cancer progression requires consideration of the type(s) of signaling molecule(s) that the enzyme might generate. Over the last decade, AA metabolites, including epoxyeicosatrienoic acids (EETs) and HETEs, have been posited as critical modulators of cancer progression, operating together with vascular endothelial growth factor (VEGF) and other growth factors to promote cellular proliferation, neovascularization and angiogenesis, cell migration, tumor growth, and metastasis. In particular, the CYP4-derived AA metabolite, 20-HETE, has long been implicated in cellular
proliferation (Roman, 2002), often in concert with VEGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF).

Similar to many CYP4 enzymes, expression of several typical EET-generating enzymes, (e.g. CYP2C and CYP2J), is upregulated in tumor cells of human origin (Wang & Dubois, 2010). Interestingly, during cancer progression, EETs formed by CYP2C and CYP2J appear to play rather similar roles to 20-HETE, in that they also stimulate tumor cell proliferation, inhibition of apoptosis, angiogenesis, and metastases in a variety of mouse tumors (Tacconelli & Patrignani, 2014). EETs also appear to participate in signaling pathways that mirror several of those invoked for 20-HETE's pro-tumorigenic effects including VEGF, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt). Administration of synthetic EET analogs or soluble epoxide hydrolase (sEH) inhibitors recapitulated these effects, whereas EET antagonists or chemical inhibitors reduced the effects on tumor size and metastases (Jiang et al., 2007; Panigrahy et al., 2011). As noted in the following section, CYP4Z1 is an EET synthase, but additional mechanisms have been proposed to explain the role of upregulated CYP4Z1 in breast cancer.

CYP4Z1 and its transcribed pseudogene, CYP4Z2P, are localized to chromosome 1p33 in a head-to-head orientation (Rieger et al., 2004). Many such genes, including CYP4Z1, have the potential to be regulated by competing endogenous RNA (ceRNA) networks, wherein pseudogene RNAs can act as a ‘sponge’ by competitive binding to common regulatory miRNA and thereby modulate expression of the parental gene (An et al., 2017). Zheng et al. (2015) first reported that 3'-UTR of CYP4Z2P promotes breast cancer angiogenesis by acting as a ceRNA for CYP4Z1 (Zheng et al., 2015), and this network has been implicated further in tamoxifen resistance in breast cancer (Zheng et al., 2016). Autoantibody generation in breast cancer has been examined by Bureik and colleagues.

Recently, the absence of a CYP4Z1 gene in lower animals has been exploited to create a human transgenic mouse model. Overexpression of CYP4Z1 in lactating female transgenic mice did not result in tumor formation or other mammary abnormalities, but upregulated estrogen receptor alpha (ERα) expression markedly (Khayeka-Wandabwa et al., 2022). In addition, these workers demonstrated a modest but statistically significant increase of ERα mRNA expression in a modified human breast cancer cell line and speculated that human CYP4Z1 might metabolize an (endogenous) small molecule into a transcriptional activator of ERα.

**CYP4Z1 substrate and metabolite profiles**

The substrate profile of CYP4Z1 continues to be limited. Saturated and unsaturated fatty acids have garnered the most interest, especially given some of the metabolites’ impact as active signaling ligands. A major point of debate has surrounded the catalytic regioselectivity of CYP4Z1. Namely, whether the enzyme follows similar CYP4 family members without a covalently linked heme to prefer internal sites of oxidation, or other CYP4 family members with a covalently linked heme that favor oxidation at the terminal position. It is important to note that the only reported xenobiotic substrates of this enzyme are pro-luciferins (Yan et al., 2017; Liu et al., 2021), and technically the mechanism-based inactivator (MBI) compounds that are covered later in this review. A more general role for CYP4Z1 as a xenobiotic-metabolizing enzyme, potentially affecting breast cancer treatments, has not been studied to our knowledge. While CYP4Z1 can be classified as an internal fatty-acid hydroxylase, both its endogenous role beyond a breast cancer association and the identification of potentially important new substrates, remain to be determined.

The first functional characterization of CYP4Z1 did not occur until 2009. Zöllner et al. utilized the fission yeast *Schizosaccharomyces pombe* and co-expressed both CYP4Z1 and CPR to probe hydroxylase activity towards saturated fatty acids commonly found in human breast tissue (Zöllner et al., 2009). In these studies, CYP4Z1 was observed to favor the in-chain ω-4 and ω-3 positions of LA and MA, respectively, in contrast to the well-known CYP4 family oxidation proclivity for terminal (ω) hydroxylation. This group also noted the
peculiar similarity of CYP4Z1-mediated metabolism patterns for medium chain fatty acid substrates to that of Jerusalem artichoke CYP81B1 (Cabello-Hurtado et al., 1998). The work of Zöllner et al. was a prequel to a number of metabolic studies through the 2010s that interrogated CYP4Z1 product specificity of fatty acid metabolites.

Firstly, Yu et al. studied the effects of CYP4Z1 overexpression in the modified breast cancer cell lines T47D and BT-474. In addition to assaying for both saturated and unsaturated fatty acids in these cells, CYP4Z1 metabolism was also suggested to be involved in generating the active signaling molecule, and terminal hydroxy product, 20-HETE. The previously studied substrates, LA and MA, and a new analyte 11,14-eicosadienoic acid, were observed to decrease in the CYP4Z1 expressing cells compared to control (indicative of metabolism). Importantly, 20-HETE content was higher in the CYP4Z1-expressing cells relative to control, although absolute quantitative values were not provided (Yu et al., 2012). CYP4Z1-dependent 20-HETE production was postulated to drive the proliferative, angiogenic, and tumor growth effects that were observed. However, these authors did not report on other putative internally positioned metabolites of AA that may have been present in their matrices.

Utilizing CYP4Z1 expressed in yeast microsomes and a robust bioanalytical method, McDonald et al. quantitated NADPH-dependent products arising from metabolism of LA and AA. Internal monohydroxy LA metabolites were formed at rates up to ~40 pmol/pmol CYP4Z1/min (favoring the ω-2 and ω-4 positions), whereas the terminal 12-OH product was not detected. AA turnover produced 14,15-EET as the clear major metabolite, with minor amounts of 19-HETE present. However, neither 20-HETE or the potential sequential oxidation product 20-carboxy-AA was detected in this system; the authors noting a detection limit for 20-HETE of 50 fmol on column (McDonald et al., 2017). Additionally, Kowalski et al. confirmed CYP4Z1’s preference for internal oxidation of AA in T47D whole cells and in HepG2 membranes engineered to express CYP4Z1. Both 14,15-EET and 14,15-dihydroxy eicosatrienoic acid (14,15-DiHET), the resultant hydrolysis metabolite, were major products, with 19-HETE as a minor product. Only a trace amount of 20-HETE was detected compared to 14,15-EET and 14,15-DiHET (Kowalski et al., 2020). In sum, these data better agree with that of Zöllner et al., where indeed CYP4Z1 is distinct from other CYP4 enzymes in its internal product regioselectivity, providing strong evidence that 20-HETE is not the major AA metabolite formed.

Beyond analysis of endogenous and technically cumbersome fatty acid substrates, further expansion of the CYP4Z1 substrate library for enzymological characterization has also progressed, notably from Prof Burek group’s use of CYP4Z1-expressing permeabilized recombinant fission yeast. Fifteen pro-luciferin compounds from Promega’s catalog of luminogenic CYP substrates were profiled using convenient P450-Glo Assays (Yan et al., 2017). CYP4Z1 was able to catalyze 11 O-dealkylation cleavages and 2 hydroxylation reactions, resulting in 13 new pro-luciferin substrates for the enzyme. Luciferin-benzyl ether (Luc-BE), luciferin-6‘-chloroethyl ether, luciferin-4F2/3, and luciferin-4F12 (Luc-4F12) were identified as the highest fidelity probes for CYP4Z1 activity, with significantly greater turnover compared to the parental control yeast strain. The sites of oxidation leading to cleavage for these substrates may be visually approximated as akin to the ω-2 to ω-6 positions of a fatty acid. Although rates were not determined for the pro-luciferins in these enzyme bags, a separate study utilizing HepG2 membranes assessed the kinetics of CYP4Z1-mediated Luc-BE O-dealkylation. A $K_m$ of 29 µM and maximal turnover of 20/min were obtained, with the enzyme following Michaelis-Menten kinetics at the substrate concentrations tested (Kowalski et al., 2020). The cornucopia of pro-luciferin substrates for CYP4Z1 has been further expanded with three fluorinated congeners of Luc-BE, three furanyl analogs, and one thiophene variant. When screened as substrates for twelve different CYP4 family enzymes, Liu et al. reported that all seven compounds were preferentially metabolized by CYP4Z1 (Liu et al., 2021).

Subsequent rationalization of pro-luciferin substrate affinity for CYP4Z1 utilized homology models based on the solved crystal structure for CYP4B1 (Hsu et al., 2017). Through docking experiments, Yan et al. postulated that active site Ser113, Ser222, Ser383, and Asn381 engage in important hydrogen bonding interactions with the carboxylate moiety, and a hydrophobic pocket engulfed the benzothiazole of pro-luciferins (Yan et al., 2017). These determinants of CYP4Z1 substrate recognition were further refined through
extensive, site-directed mutagenesis, enzyme activity, and modelling work. While the roles of the aforementioned Ser residues appeared more minimal than expected, Asn381 and Arg487 emerged as key active site residues. Du et al. theorized that these amino acids engage in hydrogen-bonding and long-range salt-bridge interactions, respectively, for carboxylate-containing substrates. Lastly, attempts to promote autocatalytic covalent heme linkage through an Ala314Glu mutation, for potential gain of ω-hydroxylation function, resulted in complete loss of enzyme activity. The authors concluded that the covalent link was not achieved, but rather the mutated Glu residue blocked substrate access to the heme (Du et al., 2020).

The identification of selective, high turnover and high through-put, probe substrates has been key to advancing a biochemical characterization of CYP4Z1. The known CYP4Z1 substrates, expression systems, resultant products, and references are summarized in Table 2.

### The evolution of CYP4Z1 inhibitors

The discovery and use of CYP4Z1 inhibitors stems from initial studies utilizing pan-CYP inhibitors to confirm the enzyme’s fatty-acid hydroxylase activity. To improve potency and selectivity, multiple groups have pursued different strategies to explore chemical space. After commercially available imidazole-containing analogs were investigated early on (Yan et al., 2017), the next generation of CYP4Z1 inhibitors evolved. Their discovery incorporated elements of substrate oxidation regioselectivity, in silico library screening utilizing CYP4Z1 homology/pharmacophore models, and new analogs of pan-CYP inhibitors generated through a lens of preferred substrate properties.

A pivotal study in the CYP4 field was the discovery of N-hydroxy-N-((butyl-2-methylphenyl) formamidine (HET0016) as a highly potent 20-HETE synthase inhibitor (Miyata et al., 2001). Miyata et al. observed potent inhibition of 20-HETE formation in rat and human renal microsomes. As four CYP4A isozymes had been reported to contribute to 20-HETE generation in the rat (Ito et al., 1998), it seemed likely that HET0016 maintains similar affinity towards these isozymes. Conversely, the potency for inhibition of EET formation in rat liver microsomes was substantially lower by ~90-fold. This early work hinted at differential HET0016 inhibitory effectiveness towards CYP isozymes that prefer terminal versus internal AA oxidation. Further research would expand the HET0016 inhibitory profile beyond the CYP4A isozymes to include members of CYP4F, CYP4V, and CYP4B families with nanomolar IC₅₀ values (Edson and Rettie, 2013; Nakano et al., 2009; Kehl et al., 2002; Parkinson et al., 2013). In aggregate, these data suggested the utility of HET0016 as a potent pan-CYP4 inhibitor, and therefore, use as a tool in CYP4Z1 studies.

To complement the previously described LA, MA, and AA metabolism studies in whole cells, Yu et al. incorporated HET0016 inhibition profiles in microsomes from CYP4Z1-expressing T47D cells. Aside from relatively weak inhibition of LA 7-hydroxylase inhibition, curiously potent inhibition of LA and MA monohydroxylation was observed, with the most pronounced effect against 20-HETE production (Yu et al., 2012). However, as part of our characterization of this orphan enzyme, we determined that HET0016 only weakly inhibited CYP4Z1 (McDonald et al., 2017). These data suggest that the inhibitor profile of HET0016 for CYP4Z1 better fits with those CYP4 enzymes without a covalently linked heme, where potency is significantly abrogated.

Yan et al. employed their newly identified pro-luciferin substrate Luc-4F12 to screen several known imidazole- and triazole-containing CYP inhibitors (Ortiz de Montellano, 2005), in CYP4Z1 yeast enzyme bags. While most compounds displayed only modest inhibition, 1-benzylimidazole (1BI) was a relatively potent inhibitor with ~29-fold improvement over HET0016 (Yan et al., 2017). Unfortunately, the utility of 1BI is limited by its inhibitory profile against other CYPs, as is common for structurally similar compounds with imidazole motifs (Wilkerson et al., 1974; Lucas et al., 1983; Ortiz de Montellano, 2005; Yan et al., 2017), and as a pan-CYP inducing agent (Magdalou et al., 1988; Mori et al., 1993; Mori et al., 2001).
By leveraging the internal regioselectivity of CYP4Z1 oxidation, the first selective and potent MBI was reported by Kowalski et al. in 2020. Design strategy was centered on the fact that an irreversible inhibitor can afford various potential benefits regarding biochemical duration, efficiency, and inactivation (Johnson et al., 2010). The pan-CYP MBI 1-aminobenzotriazole (ABT) was chosen as a bioactivatable pharmacophore, with positioning of ABT oxidation internally to match the enzyme’s regioselectivity for fatty acid hydroxylation. The 8-carbon tail analog, 8-[1H-benzotriazol-1-yl]amino)octanoic acid (8-BOA), was found to be ~60-fold more potent than ABT itself towards CYP4Z1 (when time-dependency was accounted for). (Kowalski et al., 2020). 8-BOA was also thoroughly counter-screened against other CYP4 family members and hepatic CYPs involved in xenobiotic metabolism that had EET-synthase activity. Both reversible (within the timeframe of the assay), and time-dependent inhibition were assessed for these ‘off-targets’. While reversible inhibition against these CYPs was low, the time-dependent inhibition (TDI) specificity ratios (off-target shifted-IC50/CYP4Z1 shifted-IC50) ranged from 230 – 2235 and 275 – 1650 for CYP4 and other hepatic CYPs, respectively, indicating exquisite selectivity when accounting for bioactivation. CYP-mediated bioactivation of ABT results in a well-characterized bridged porphyrin adduct via formation of reactive benzyne (Ortiz de Montellano & Mathews, 1981). Initial studies demonstrated that while ABT itself formed the prototypical adduct with CYP4Z1 heme, this was not observed for 8-BOA and, therefore, its mechanism of CYP4Z1 inactivation remains unknown (Pelletier et al., 2023).

To evaluate the utility of 8-BOA in an in vivo setting, pharmacokinetics and circulating metabolites were next determined in the rat (Kowalski et al., 2021). Relatively low clearance, a moderate volume of distribution, and somewhat prolonged half-life were noted. Most interestingly, the major metabolites in plasma were products of β-oxidation, with the -(CH$_2$)$_2$ metabolite also characterized as a CYP4Z1 MBI. It was hypothesized that this active metabolite might even enhance the inhibitory effect of 8-BOA if the parent compound were to be used in animal models to probe CYP4Z1-mediated tumor growth.

The development of non-covalent CYP4Z1 inhibitors progressed substantially from the efforts of Machalz et al. who drew on their initial data with the inhibitor 1BI, updated CYP4Z1 homology modeling, and expansive in silico efforts. Starting with a virtual library of ~10 million compounds, five commercially available candidates were chosen to assess that met requirements for affinity, heme-azole coordination (desired motif), and an Arg487 salt bridge. In CYP4Z1-overexpressing MCF-7 cells, 3-[(4-(1H-imidazol-1-yl)benzamido)methyl]benzoic acid (inhibitor 9), an imidazole linked via N-benzylbenzamide to a carboxylate moiety, was highly potent and ~100-fold more potent than its predecessor 1BI in this assay system. Also assessed was the CYP5A1 inhibitor Ozagrel, which is used in the treatment of asthma (Iizuki et al., 1981), but its potency towards CYP4Z1 was low, the time-dependent inhibition (TDI) specificity ratios (off-target shifted-IC50/CYP4Z1 shifted-IC50) ranged from 230 – 2235 and 275 – 1650 for CYP4 and other hepatic CYPs, respectively, indicating exquisite selectivity when accounting for bioactivation. CYP-mediated bioactivation of ABT results in a well-characterized bridged porphyrin adduct via formation of reactive benzyne (Ortiz de Montellano & Mathews, 1981).

The most recently reported development in CYP4Z1 inhibitors is an extensive profiling of new HET0016 derivatives generated by Yuan et al. in 2022. An analog modified with an esterified 4-carbon carboxylate tail, ethyl (E)-4-[(4-(N-hydroxyformimidamido)phenyl)butanoate (7c), resulted in a ~14-fold improvement over HET0016 in their assay system. The suggested 7c binding mode in their CYP4Z1 homology model implicates Arg384 and Ser383 as engaging in hydrogen bonding with the carbonyl oxygen, with expected coordination of the Fe atom via the hydroxyformamidine moiety. Excellent selectivity of 7c for 4/5 off-targets indicated the enzyme’s regioselectivity for fatty acid hydroxylation. The (CH$_2$)$_2$ metabolite also characterized as a CYP4Z1 MBI. It was hypothesized that this active metabolite might even enhance the inhibitory effect of 8-BOA if the parent compound were to be used in animal models to probe CYP4Z1-mediated tumor growth.

The authors did note a limitation for in vivo use in that rapid hydrolysis of 7c occurs from esterase(s) in mouse plasma and liver microsomes, with observed t$_{1/2}$ values of ~25 and ~13 min, respectively. Although it would be an active metabolite, the formed carboxylate congener was interestingly reported to have much lower potency towards CYP4Z1. Extensive research into the contribution of CYP4Z1 towards breast cancer cell stemness, and the efficacy of 7c in attenuating this property, was also pursued by this group. In brief, when MCF-7 and MDA-MB-231 cells were treated with 7c, the metastatic ability, spheroid formation, and expression of stemness markers were all lowered. Furthermore, when these same cell lines were pretreated with 7c and implanted
immunodeficient mice, tumor-initiating ability was somewhat abrogated (although tumor weights were not significantly different).

The promising results described by Yuan et al. appear to lend credence to the hypothesis that CYP4Z1 is a driver of certain cancers. However, while CYP4Z1 overexpression has been employed to generate lysates for inhibitor profiling, no experimental information on the levels (i.e., pmol/million cells) of cellular expression of CYP4Z1 in these studies is available. Notably, several immortalized breast cancer cell lines (including MCF-7) are reported to have lost constitutive expression of CYP4Z1, and the protein is not detectable in the parental lines (Rieger et al., 2004; Savas et al., 2005). Furthermore, it seems well established that these cell lines are catalytically insufficient to support metabolism studies without CYP4Z1 overexpression (Yu et al., 2012; Kowalski et al., 2020). Therefore, while it cannot be excluded that inhibition of even minimal CYP4Z1 activity in these breast cancer cell lines might be enough to elicit the measurable responses that were reported, more experimental data to address these questions is desirable.

The inhibitors discussed above have already proven useful in biochemical characterization of CYP4Z1 and will likely have utility in future CYP4Z1 research. However, it is important to note that with the different CYP4Z1 expression systems and probe substrates/concentrations utilized, direct potency comparisons between inhibitors should be taken with care. Benchmarking against internal data for a commonly used control inhibitor such as HET0016 (which spans a ~50-fold range between research groups) can provide some clarity on the relative potency differences. The known CYP4Z1 inhibitors, potencies, and references are summarized in Table 3.

**Cancer-specific cytotoxicity: Identifying protoxins for CYP4Z1 bioactivation**

In their report on its identification, Rieger et al. suggested utilizing the tissue- and cancer-specific expression of CYP4Z1 to bioactivate prodrugs into toxins for the treatment of breast carcinomas. Indeed, there are successful anticancer agents in the clinic that require metabolic activation from CYPs, two well-known examples being cyclophosphamide and ifosfamide (Ortiz de Montellano, 2013). CYP4Z1 protoxins could potentially have both high efficacy and therapeutic index. This strategy has been echoed by a number of groups since (including our own), however, nothing has come to fruition yet. Furthermore, pursuing this angle is not dependent on CYP4Z1 being validated as a target that drives breast cancer itself.

Seemingly juxtaposed to twenty years without reported progress into protoxin discovery for CYP4Z1, the fellow family member CYP4B1 is rife with relatively well-characterized furan- and cyclic amine-containing protoxin substrates, dating back to initial work in the 1970s (Boyd, 1976; Slaughter et al., 1983; Czerwinski et al., 1991; Baer and Rettie, 2006; Roellecke et al., 2017). Although predominantly expressed in the lung (potentially allowing targeted lung cancer treatment), the human ortholog is non-functional which impedes successful application in cancer treatment without introducing a re-engineered animal/chimeric analog (Rowinsky et al., 1993; Wiek et al., 2015). As such, CYP4B1 has been proposed as part of suicide gene systems for targeted cancer therapy (Röder et al., 2023).

While it is unknown how many groups have actually pursued CYP4Z1 protoxin research, if attempts have failed, perhaps structural differences in the enzyme active site, heme linkage, and/or substrate access channel are the culprit. We hypothesized that the relatively hydrophobic CYP4B1 active site, which seemingly envelops hydrophobic substrates for efficient bioactivation, can readily eject reactive electrophiles with consequent tissue nucleophile targeting and toxicity (Kowalski et al., 2019). Perhaps positioning above the heme of CYP4Z1 and/or relatively tight interaction geometries for substrates, stemming from the enzyme’s recognition sites (Du et al., 2020), prohibit structural motifs prone to bioactivation from efficient binding. Alternatively, CYP4Z1-bioactivatable compounds could potentially be missed due to enzyme inactivation occurring instead, where reactive intermediates modify the apoprotein and/or destroy function of the heme. For the MBI we reported on, a comparatively low partitioning of inactivator molecules escaping the enzyme active...
site relative to catalytic cycle inactivation was observed (Kowalski et al., 2020a). Perhaps the issue is related to reactive metabolites not escaping the CYP4Z1 active site to elicit cytotoxicity? Regardless, if discovered, a CYP4Z1 protoxin may prove to have wide-ranging impact in the breast cancer treatment field.

**Purification of CYP4Z1 and solving the crystal structure**

Varied overexpression systems have been successfully utilized to characterize CYP4Z1 regarding both its catalytic and (potential) cancer-promoting functions. As discussed above, these have included microsomes/membranes of fission yeast strains, permeabilized yeast enzyme bags, HepG2/HEK293T membranes, and the breast cancer cell lines T47D/MCF-7/MDA-MB-231/BT-474. However, a notable omission in the CYP4Z1 research field is the use of purified enzyme for biochemical characterization.

During the graduate thesis work of one of us (JPK) we attempted the recombinant expression and purification of CYP4Z1 from the yeast *Saccharomyces cerevisiae* (Kowalski, 2020b). We were successful in isolating a small amount of highly pure CYP4Z1 ([Figure 2A, B](#)) that provided a carbon-monoxide binding spectrum indicative of mostly holo-enzyme, *i.e.* with functional heme ([Figure 2C](#)). Using a reconstituted system and semi-quantitative liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection, an assessment of the capability of purified CYP4Z1 to metabolize AA was undertaken. With the purified reconstituted enzyme, we confirmed that CYP4Z1 generates 14,15-EET >60-fold more than 20-HETE, where only a trace amount could be detected ([Figure 2D](#)). This metabolite preference matched what our group has observed in numerous other CYP4Z1-expression systems but also lends support to some, albeit very minimal, ability to generate 20-HETE as reported by Yu et al. However, as mentioned above we were continually plagued by extremely low yield, due in large part to poor detergent solubilization and binding to the nickel nitritetriacetic (Ni-NTA) column. Cloning new expression vectors with truncated (N-terminus) CYP4Z1 variants to enhance solubilization, assessing different detergents to boost membrane extraction, and improving Ni-NTA column binding through extended His- or glutathione S-transferase-tags may be beneficial for improving yield.

As previously discussed, significant progress has been made in the generation of homology models of CYP4Z1 that utilize the recently solved crystal structure of CYP4B1 (Hsu et al., 2017; Yan et al., 2017; Du et al., 2020). In general, catalytic activities of expressed CYP4Z1 mutant variants trend with active site/substrate channel residue mutations that are modeled to abrogate positioning above the heme and/or substrate affinity. However, a major gap in CYP4Z1 research is the lack of a solved crystal structure. While the previously discussed challenge of expressing and purifying CYP4Z1 must of course be tackled first. Such a tool may enable exciting discoveries in the CYP4Z1 field regrading additional endogenous substrates (and their potential roles in breast cancer progression), novel inhibitors, bioactivatable protoxins, and structural inferences for other CYP4 family enzymes.

**Concluding Remarks**

Early studies on the role of CYPs in cancer date back more than 50 years when the enzymes’ metabolic activity was implicated in the bioactivation of nitrosamines to mutagenic metabolites(s) (Czygan et al., 1973). In the late 1980s, a series of studies by Waxman and colleagues established the primal role of CYP enzymes in bioactivating key cancer drugs like cyclophosphamide and thiotepa (Clarke & Waxman, 1989; Teicher et al., 1989). In the 1990s, a prodrug activation strategy was outlined by Rainov, Philpot and colleagues that used rabbit CYP4B1 along with the furan pro-toxin 4-ipomeanol that is bioactivated to cytotoxic metabolites, which can kill cancer cells (Rainov et al., 1998). This ‘suicide gene’ strategy was further refined recently by Hanenberg's group for application in adoptive T-cell therapies using a highly active modified human CYP4B1 (Roellecke et al., 2016). Today, attention in this area has turned increasingly towards CYP4Z1 as the target for enzyme inhibitors (Kowalski et al., 2020a; Machalz et al., 2021; Yuan et al., 2022), and potentially as a protoxin activating CYP in breast cancer. There and back again indeed!
Data Availability Statement

This review article contains no datasets generated or analyzed during the current study beyond those widely available from The Human Protein Atlas: https://www.proteinatlas.org/
References


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Figure 1. Distribution of CYP4Z1 mRNA in normal and cancer tissues

Figure 2. Recombinantly Expressed and Isolated CYP4Z1

Table 1. Characteristics of Human CYP4 Enzymes

Table 2. CYP4Z1 Substrates

Table 3. Notable CYP4Z1 Inhibitors
**Figure 1.** Distribution of CYP4Z1 mRNA in normal (A) and cancer (B) tissues.


**Figure 2.** Recombinantly Expressed and Isolated CYP4Z1

Characterization of recombinantly expressed and isolated CYP4Z1, with LC-MS/MS analysis of CYP4Z1 metabolism of AA. CYP4Z1 was expressed in *Saccharomyces cerevisiae* (Y12) and purified using affinity chromatography (Ni-NTA). In SDS-PAGE (silver stain detection) analysis of the purified enzyme no contaminating proteins were observed, only CYP4Z1 migrating at ~ 55 kDa (A). Western blot of the isolated enzyme with anti-CYP4Z1 (Atlas Antibodies) (B). Carbon-monoxide binding spectra obtained with purified CYP4Z1, a λ<sub>max</sub> of 448 nm was observed (C). The metabolism of AA (50 µM) in a reconstituted system with recombinantly expressed and purified CYP4Z1 (250 nM) was NADPH-dependent and preferentially produced 14,15-EET (highlighted in red). Equal amounts of the internal standards 20-HETE-d₆ and 14,15-EET-d₁₁ (100 ng) were added during sample workup to enable semi-quantitative comparisons of metabolite formation for these two analytes. Note, due to lack of chemical standards for 16–19-HETE, the assignments presented here for these metabolites are tentative (D). Expression and reconstitution methods generally followed those outlined by Gillam et al., 1993 and Pompon et al., 1994; LC-MS/MS methods may be found in McDonald et al., 2017.
<table>
<thead>
<tr>
<th>Sub-family</th>
<th>Enzyme</th>
<th>Localization</th>
<th>Covalent Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP4A</td>
<td>4A11</td>
<td>Liver, kidney</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td></td>
<td>4A22</td>
<td>Liver, kidney</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td>CYP4B</td>
<td>4B1</td>
<td>Lung, bladder</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td>CYP4F</td>
<td>4F2</td>
<td>Liver, kidney</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td></td>
<td>4F3A</td>
<td>Myeloid tissues</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td></td>
<td>4F3B</td>
<td>Liver, kidney</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td></td>
<td>4F8</td>
<td>Urogenital</td>
<td>No (FGGHDT)</td>
</tr>
<tr>
<td></td>
<td>4F11</td>
<td>Liver, kidney</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td></td>
<td>4F12</td>
<td>Liver, intestine</td>
<td>No (FGGHDT)</td>
</tr>
<tr>
<td></td>
<td>4F22</td>
<td>Skin</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td>CYP4V</td>
<td>4V2</td>
<td>Retina</td>
<td>Yes (FEGHDT)</td>
</tr>
<tr>
<td>CYP4X</td>
<td>4X1</td>
<td>Brain, skin</td>
<td>No (LAGHDT)</td>
</tr>
<tr>
<td>CYP4Z</td>
<td>4Z1</td>
<td>Breast tissue</td>
<td>No (FAHGDT)</td>
</tr>
</tbody>
</table>

Adapted from McDonald et al., 2017.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Metabolites</th>
<th>Expression System</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauric acid (LA)</td>
<td><img src="image" alt="Structure" /></td>
<td>8-OH &gt; 7-, 9-, 10-OH 7-, 8-, 9-, 10-OH* 8-OH &gt;&gt; 10-OH &gt; 11-OH &gt; 9-OH &gt; 7-OH</td>
<td>Yeast whole cells</td>
<td>Zollner et al., 2009</td>
</tr>
<tr>
<td>Myristic acid (MA)</td>
<td><img src="image" alt="Structure" /></td>
<td>12-OH &gt; 11-, 10-, 9-OH 9-, 10-, 11-, 12-OH*</td>
<td>Yeast whole cells</td>
<td>Zollner et al., 2009</td>
</tr>
<tr>
<td>11,14-Eicosadienoic acid</td>
<td><img src="image" alt="Structure" /></td>
<td>Not reported (depletion observed)</td>
<td>T47D whole cells</td>
<td>Yu et al., 2012</td>
</tr>
<tr>
<td>Arachidonic acid (AA)</td>
<td><img src="image" alt="Structure" /></td>
<td>20-HETE 14,15-EET &gt;&gt; 19-HETE 14,15-EET &gt;&gt; 14,15-DiHET &gt;&gt; 19-HETE &gt;&gt; 20-HETE (trace)</td>
<td>T47D microsomes</td>
<td>Yu et al., 2012</td>
</tr>
<tr>
<td>Luciferin-benzyl ether (Luc-BE)</td>
<td><img src="image" alt="Structure" /></td>
<td>Luciferin</td>
<td>Yeast enzyme bags</td>
<td>Yan et al., 2017*</td>
</tr>
<tr>
<td>Luciferin-4F12 (Luc-4F12)</td>
<td><img src="image" alt="Structure" /></td>
<td>Luciferin</td>
<td>Yeast enzyme bags</td>
<td>Liu et al., 2021*</td>
</tr>
<tr>
<td>Luciferin-3-fluorobenzyl ether (Luc-3FBE)</td>
<td><img src="image" alt="Structure" /></td>
<td>Luciferin</td>
<td>Yeast enzyme bags</td>
<td></td>
</tr>
<tr>
<td>Luciferin-4-fluorobenzyl ether (Luc-4FBE)</td>
<td><img src="image" alt="Structure" /></td>
<td>Luciferin</td>
<td>Yeast enzyme bags</td>
<td></td>
</tr>
</tbody>
</table>
No relative ranking of product formation reported.

All matrices from these studies showed this general product ranking; tentatively assigned were the metabolites 16-/17-/18-HETE.

Pro-luciferin substrates also used by multiple groups for activity/inhibitor screening.

Additional pro-luciferin probe substrates reported, those listed here showed highest CYP4Z1 activity.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure</th>
<th>IC₅₀ (nM)</th>
<th>Relative Potency</th>
<th>Probe Substrate</th>
<th>Expression System</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Hydroxy-N-(butyl-2-methylphenyl)-formamidine (HET0016)</td>
<td></td>
<td>2,175; 322; 523; 168⁶ 286; 549; 241; 169⁵ 29.8⁴</td>
<td>-</td>
<td>LA MA AA</td>
<td>T47B microsomes</td>
<td>Yu et al., 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~15,000⁵</td>
<td>-</td>
<td>LA (100 µM)</td>
<td>Yeast microsomes</td>
<td>McDonald et al., 2017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~5,200⁶</td>
<td>-</td>
<td>Luc-4F12⁷ (10 µM)</td>
<td>Yeast enzyme bags</td>
<td>Yan et al., 2017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>601</td>
<td>-</td>
<td>Luc-BE⁸ (50 µM)</td>
<td>HEK293T lysates</td>
<td>Yuan et al., 2022</td>
</tr>
<tr>
<td>1-Benzylimidazole (1-BI)</td>
<td></td>
<td>180</td>
<td>29x</td>
<td>Luc-4F12 (10 µM)</td>
<td>Yeast enzyme bags</td>
<td>Yan et al., 2017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5,900</td>
<td>0.9x</td>
<td>Luc-BE (150 µM)</td>
<td>MCF-7 whole cells</td>
<td>Machalz et al., 2021</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6,700</td>
<td>0.8x</td>
<td>Luc-3FBE⁹ (150 µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Aminobenzotriazole (ABT)</td>
<td></td>
<td>~25,000⁵</td>
<td>0.2x</td>
<td>Luc-4F12 (10 µM)</td>
<td>Yeast enzyme bags</td>
<td>Yan et al., 2017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>154,000</td>
<td>0.1x</td>
<td>Luc-BE (29 µM)</td>
<td>HepG2 membranes</td>
<td>Kowalski et al., 2020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12,000 (shifted)⁶</td>
<td>1.3x</td>
<td>Luc-3FBE⁹ (150 µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-[1H-benzotriazol-1-yl]amino]octanoic acid (8-BOA)</td>
<td></td>
<td>5,900</td>
<td>2.5x</td>
<td>Luc-BE (29 µM)</td>
<td>HepG2 membranes</td>
<td>Kowalski et al., 2020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 (shifted)⁷</td>
<td>75x</td>
<td>Luc-3FBE⁹ (150 µM)</td>
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</tr>
<tr>
<td>3-[(4-(1H-imidazol-1-yl)benzamido)methyl]benzoic acid (inhibitor 9)</td>
<td></td>
<td>63</td>
<td>83x</td>
<td>Luc-3FBE (150 µM)</td>
<td>MCF-7 whole cells</td>
<td>Machalz et al., 2021</td>
</tr>
<tr>
<td>Ethyl (E)-4-[(4-(N-hydroxyformimidamido)phenyl)butanoate (7c)</td>
<td>42</td>
<td>14x</td>
<td>Luc-BE (50 µM)</td>
<td>HEK293T lysates</td>
<td>Yuan et al., 2022</td>
<td></td>
</tr>
<tr>
<td>-----</td>
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</tr>
</tbody>
</table>

- Fold change in potency (HET0016 IC\(_{50}\) / Inhibitor IC\(_{50}\)) compared to each research group’s internal characterization, with matched probe substrate when available, and HET0016 IC\(_{50}\) values were utilized for Yan et al., 2017 & Machalz et al., 2021; Kowalski et al., 2020; and Yuan et al., 2022 comparisons, respectively.
- IC\(_{50}\) values refer to inhibition of specific CYP4Z1 hydroxylase activities towards LA: 7-, 8-, 9-, 10-OH, and MA: 9-, 10-, 11-, 12-OH, in the respective orders listed; only inhibition of 20-HETE formation was assessed for AA as probe substrate; probe substrate concentrations for LA, MA, and AA were not reported.
- HET0016 inhibition towards CYP4Z1-mediated LA 8-, 9-, 10-OH activities ranged from 14.7 – 15.9 µM and averaged ~15 µM.
- IC\(_{50}\) towards Luc-4F12 metabolism for HET0016 and ABT extrapolated from single point 10 µM ~IC\(_{66}\) and ~IC\(_{29}\) values, respectively, assuming a Hill slope of 1.
- For all pro-luciferin probe substrates (Luc-4F12, Luc-BE, Luc-3FBE), inhibition of O-dealkylation to the luciferin product was monitored.
- Shifted-IC\(_{50}\) incorporated a 30 min pre-incubation step to evaluate time-dependent inhibition; for 8-BOA, a \(k_{inact} = 2.2 \mu M\) and \(k_{inact} = 0.15 \text{min}^{-1}\) were also reported.
- Luc-BE utilized at the determined CYP4Z1 \(K_m\) in this over-expression system.
Figure 2.

A

B

C

Absorbance

0.000
0.005
0.010
0.015
0.020
Wavelength (nm)

420
440
460
480
500

D

1: 19-HETE
2: 20-HETE-\text{d}_6
3: 18-HETE
4: 20-HETE (trace)
5: 17-HETE
6: 16-HETE
7: 14,15-EET-\text{d}_{11}
8: 14,15-EET