Deciphering the role of fatty acid metabolizing CYP4F11 in lung cancer and its potential as a drug target

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CPR: NADPH-dependent cytochrome P450 reductase; P450: cytochrome P450
Abstract

Lung cancer is the leading cause of cancer deaths worldwide. We found that the cytochrome P450 isoform CYP4F11 is significantly overexpressed in patients with lung squamous cell carcinoma. CYP4F11 is a fatty acid ω-hydroxylase and catalyzes the production of the lipid mediator 20-hydroxyeicosatetraenoic acid (20-HETE) from arachidonic acid. 20-HETE promotes cell proliferation and migration in cancer. An inhibition of 20-HETE-generating cytochrome P450 enzymes has been implicated as novel cancer therapy for more than a decade. However, the exact role of CYP4F11 and its potential as drug target for lung cancer therapy has not been established yet. Thus, we performed a transient knockdown of CYP4F11 in the lung cancer cell line NCI-H460. Knockdown of CYP4F11 significantly inhibits the lung cancer cell proliferation and migration while the 20-HETE production is significantly reduced. For biochemical characterization of CYP4F11-inhibitor interactions, we generated recombinant human CYP4F11. Spectroscopic ligand binding assays were conducted to evaluate CYP4F11 binding to the unselective CYP4A/F inhibitor HET0016. HET0016 shows high affinity to recombinant CYP4F11 and inhibits CYP4F11-mediated 20-HETE production in vitro with a nanomolar IC$_{50}$. Cross evaluation of HET0016 in NCI-H460 cells shows that lung cancer cell proliferation is significantly reduced together with 20-HETE production. However, HET0016 also displays antiproliferative effects which are not 20-HETE mediated. Future studies aim to establish the role of CYP4F11 in lung cancer and the underlying mechanism and investigate the potential of CYP4F11 as a therapeutic target for lung cancer.
Significance Statement

Lung cancer is a deadly cancer with limited treatment options. The cytochrome P450 4F11 (CYP4F11) is significantly upregulated in lung squamous cell carcinoma. A knockdown of CYP4F11 in a lung cancer cell line significantly attenuates cell proliferation and migration with reduced production of the lipid mediator 20-HETE. Studies with the unselective inhibitor HET0016 shows a high inhibitory potency of CYP4F11-mediated 20-HETE production using recombinant enzyme. Overall, our studies show the potential of targeting CYP4F11 for new transformative lung cancer treatment.
Introduction

Lung cancer is the leading cause of cancer related deaths worldwide with only limited treatment options. Most of lung cancer cases derive from excessive tobacco use which leads to a dysregulation of many genes triggering oncogenesis (Sridhar et al., 2008). Studies comparing the gene expression profiles in lung epithelial cells between smokers and non-smokers found that expression of the cytochrome P450 4F11 (CYP4F11) is significantly induced by tobacco smoke (Sridhar et al., 2008). The expression of CYP4F11 is induced by the redox master regulator Nrf2 (nuclear factor erythroid 2–related factor 2) which is upregulated in lung cancer due to increased oxidative stress (Bar-Peled et al., 2017; McMillan et al., 2018). However, the role of CYP4F11 in lung cancer progression is yet to be elucidated.

Cytochrome P450 enzymes (P450, CYP) are heme thiolate proteins and are indispensable for human life. They are the major drug and xenobiotic metabolizing enzymes and are involved in the synthesis of steroid hormones and the metabolism of fatty acids and bile acids (Guengerich, 2019). The isoform CYP4F11 belongs to the CYP4 family of fatty acid ω-hydroxylases which catalyze the metabolism of various medium to very long chain fatty acids and eicosanoids (Edson and Rettie, 2013). CYP4F11 stands out from other stricter ω-hydroxylases due to its broader substrate range. In addition to fatty acids, it can also efficiently metabolize drugs such as the macrolide antibiotic erythromycin and is the only CYP4F isoform known to metabolize 3-hydroxy fatty acids (Kalsotra et al., 2004; Dhar et al., 2008). The upregulation of CYP4F11 in lung cancer has been recently exploited for the activation of prodrugs for lung cancer treatment. CYP4F11 activates these prodrugs by O-demethylation which then target the stearoyl-CoA-desaturase (Theodoropoulos et al., 2016; Winterton et al., 2018). Most intriguingly, CYP4F11 knockdown in lung cancer cells led to a reduced colony formation in soft agar assays (Bar-Peled et al., 2017). However, fundamental studies are...
missing to shed light on CYP4F11 function in lung cancer and to assess its future use as a lung cancer drug target.

CYP4F ω-hydroxylases have been long associated with cancer progression due to their ability to catalyze the conversion of arachidonic acid to the potent lipid mediator 20-hydroxyeicosatetraenoic acid (20-HETE) (Figure 1A). In healthy individuals, 20-HETE regulates the blood pressure and promotes angiogenesis (Roman and Fan, 2018). However, in cancer, 20-HETE promotes cell proliferation and migration and tumor angiogenesis (Alexanian et al., 2012; Alexanian and Sorokin, 2013). In studies with xenograft mouse models the growth of lung cancer tumors overexpressing a 20-HETE producing CYP4 isoform was accelerated while an inhibition of 20-HETE production resulted in drastically decreased tumor growth (Yu et al., 2011; Borin et al., 2014). Here, 20-HETE triggered the expression of VGEF and MMP-9 which are key players in cell migration and invasion (Yu et al., 2011). The pro-carcinogenic effect of 20-HETE has been confirmed in other cancer types, such as breast cancer, prostate cancer, and renal adenocarcinoma (Alexanian et al., 2009; Borin et al., 2014; Borin et al., 2017; Cardenas et al., 2020).

Targeting CYP4-mediated 20-HETE production for cancer treatment has been proposed for more than a decade (Alexanian and Sorokin, 2013). Efforts to find an efficient inhibitor for 20-HETE production led to the development of the small molecule HET0016 which is a pan inhibitor of 20-HETE synthesis (Figure 1B) (Miyata et al., 2001; Borin et al., 2014). HET0016 is a powerful tool to examine the impact of 20-HETE on various disease states such as cancer, kidney fibrosis, traumatic brain injury, ischemic stroke, and cancer (Kehl et al., 2002; Borin et al., 2014; Ge et al., 2014; Colombero et al., 2017; Cui et al., 2021). Using human liver microsomes, HET0016 inhibits 20-HETE production with an IC₅₀ (half-maximal inhibitory concentration) of 8.9 nM (Miyata et al., 2001). In cancer, the use of HET0016 and subsequent decrease of 20-HETE levels led to decreased tumor growth in breast and lung cancer xenograft mouse models (Yu et al., 2011; Borin et al., 2014). However, HET0016 is an unselective
inhibitor of all 20-HETE producing P450 ω-hydroxylases which impedes its clinical use due to potential serious side effects. The various CYP4 isoforms assume different roles in the human body. CYP4F2, CYP4F3B, and CYP4A11 are considered the major 20-HETE producers in liver and kidney (Lasker et al., 2000). CYP4F2 also participates in vitamin K clearance (Edson et al., 2013). The isoform CYP4F3A is expressed in monocytes and is involved in the anti-inflammatory response by deactivating leukotriene B4 (Kikuta et al., 1998). CYP4F22 is expressed in the human skin and participates in ceramide synthesis which is crucial for maintaining the skin barrier (Ohno et al., 2015). This shows that an unselective inhibition of 20-HETE could cause tremendous side effects for the patients. Thus, due to its lack of selectivity, HET0016 is not a suitable tool to study CYP4F isoform specific contributions to cancer growth and migration.

To promote the exploitation of CYP4F enzymes as cancer drug targets, it is mandatory to elucidate which CYP4 isoforms are overexpressed in the respective cancer type for directed targeting. In addition, an isoform specific link between lung cancer oncogenesis and 20-HETE production has to be established. In the study presented here, we demonstrate that the isoform CYP4F11 plays a pivotal role in lung cancer cell growth and migration and that observed effects are associated with 20-HETE production. Using recombinant enzyme, we characterize the binding affinity and inhibitory efficiency of the CYP4 pan inhibitor HET0016 on CYP4F11-mediated 20-HETE production.
Material and Methods

Materials

Arachidonic acid, 20-hydroxyeicosatetraenoic acid (20-HETE), and HET0016 were purchased from Cayman Chemicals (Ann Arbor, MI). The lung cancer cell line NCI-H460 was purchased from ATCC. The cell line was cultured in RPMI 1640 (ATCC, 2.05 mM L-glutamine) supplemented with 10% FBS (ATCC and 1% penicillin/streptomycin (ThermoFisher)) and maintained in a humidified environment in the presence of 5% CO₂ at 37°C. Chemicals and reagents were purchased from Sigma Aldrich or comparable. A list of used siRNAs and antibodies is included in the supplementary material.

siRNA and transient transfection

Transfection in 10 cm² plate was conducted according the Lipofectamine® RNAiMAX reagent protocol (Life technologies). 6 µl siRNA (10 µM) in 225 µl of serum-free Opti-MEM medium (Gibco) was mixed with 18 µl of Lipofectamine® RNAimax reagent in 300 µl serum-free Opti-MEM medium. Following a 5 min incubation, the siRNA-lipid mixture was transferred to a 10 cm² plate followed by plating of cells at a concentration of 4×10⁶ cells/plate. Cells were transfected for 24h.

Western blot analysis

Protein extracts were harvested from NCI-H460 siCYP4F11 and control cells 72h post transfection. Cells were lysed using cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v)deoxycholic acid, and 1X Halt TM protease inhibitors cocktail (Thermo scientific)) for 20 min at 4 °C. The cell lysates were centrifuged for 20 min at 14,000g at 4°C. The protein concentration of the supernatant was determined by Pierce TM BCA Protein Assay Kit (Thermo scientific). Protein sample was denatured with 4X SDS loading buffer (200 mM Tris-HCl (pH 6.8), 8% (w/v) SDS, 40% (v/v) glycerol, 60 mM EDTA, 0.08%
bromophenol blue, and 20% (v/v) 2-mercaptoethanol) by boiling for 5 min at 100°C. Protein lysate (20 µg) was loaded on 4-20% Tris-glycine gel (Mini-PROTEAN TGX Gels, Bio-Rad) for SDS PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-FL Transfer membrane, Merk Millipore Ltd.). The membrane was incubated with CYP4F11 primary antibodies (Invitrogen) in blocking buffer (5% nonfat milk, VMR) overnight at 4°C. The membrane was then washed and incubated for 1 h at room temperature with a peroxidase-conjugated secondary antibody and imaged using Pierce TM ECL Western Blotting Substrate (Thermo scientific) and IBright 1500 imaging system (Invitrogen). The membranes were stripped and reprobed with GAPDH primary antibody (Invitrogen) that served as a loading control.

**Cell viability assay**

The siCYP4F11 cells were seeded 24 h post-transfection at the concentration of 5,000 cells/well in 96-well plates. The consequences on cell viability were determined 48 h post-transfection. The proliferation of the cells was detected by cell counting using the automated cell counter Invitrogen Countess3 (Thermo Fisher) and MTT Cell Proliferation Assay Kit (Abcam) following the protocol. The absorbance was measured at 590 nm using a microplate reader (BioTek). The data was normalized using GraphPad Prism to report cell viability [100%].

**Wound healing assays**

The NCI-H460 siControl and siCYP4F11 cells were seeded 24 h post-transfection at a concentration of 4×10^6 cells/well in 10 cm² well plate. The monolayer was washed with phosphate buffer saline (PBS) to remove cell debris, supplemented with serum-free medium for 12 h. A single scratch wound was created on the confluent monolayers using a micropipette tip (2-20 µl). The plate was incubated at 37 °C and was observed at 0h, 8 h, 12 h, and 24 h under the microscope. Migration ability of the cells was normalized based on the unmigrated area at 0 h and reported in % migration. The unmigrated area was calculated using the software imageJ.
20-HETE ELISA immunoassay

The NCI-H460 siControl and siCYP4F11 cells were seeded 24h post-transfection at a concentration of $2 \times 10^6$ cells/well in 10 cm$^2$ well plates. Experiments involving chemical treatment were designed with HET0016 treatment 48 h post-transfection for 24 h at 37°C at the indicated doses (0.1 µM, 10 µM, 100 µM). Cells were detached and washed with ice cold PBS 72h post-transfection. The extraction of 20-HETE from cells and the detection of 20-HETE production were followed according to the 20-HETE ELISA protocol (Abcam). The obtained data was normalized to cell seeding density and further normalized using GraphPad Prism to report cell viability [100%].

Generation of human recombinant CYP4F11 and NADPH-dependent cytochrome P450 reductase

Tang et al. (Tang et al., 2010) reported the first expression and purification protocol for human CYP4F11. However, we significantly altered the protocol to increase yield and purity of the enzyme using the following strategies: Use of C43(DE3) cells instead of BL21(DE3) cells, use of TB media with 2 X instead of 1 X potassium phosphate buffer to avoid culture acidification, decrease of expression temperature to 26°C, use of histidine instead of imidazole and optimization of wash step for IMAC purification, use of Tween 20 instead of Tergitol NP-10, and the use of CHAPS instead of sodium cholate which was particularly important to decrease the ionic strength for the subsequent anion exchange chromatography since CYP4F11 has a isoelectric point of 6.43 and did not readily bind to the column. We did not perform a hydroxyapatite chromatography as described in Tang et al., but further purified CYP4F11 with anion exchange chromatography to achieve higher protein purity and stability. The detailed protocol with buffer compositions is described in the following.
The cDNA for human CYP4F11 was codon optimized and modified for recombinant expression in *Escherichia coli* (*E. coli*). The N-terminal modification MALLAFVL was added to the 7th amino acid of wild type CYP4F11 and a 6x Histidine tag was added to the C-terminus to facilitate purification. The cDNA was then cloned into a pCWori+ vector, the vector was transformed into the *E. coli* strain C43(DE3) and plated on LB agar containing the pCWori+ selection marker carbenicillin (100 μg/ml). To start CYP4F11 expression, 30 mL LB medium containing carbenicillin (100 μg/ml) were inoculated with 4-5 colonies of transformed *E. coli* and grown over night shaking at 37°C. For the main culture, 400 mL TB medium (for 1 l: 24 g technical yeast extract, 12 g peptone from casein, 4 mL glycerol) buffered with 2X potassium phosphate buffer (for 100 mL 20X solution: 4.62 g K_2HPO_4, 25.08 g KH_2PO_4) were placed in six 2.8 l Fernbach flasks, respectively, and 100 μg/ml carbenicillin was added. Each flask was then inoculated with 4 ml seed culture and incubated with shaking at 37°C and 210 rpm. When an OD<sub>600</sub> of 0.5 was reached, the protein expression was induced with 1 mM IPTG and 1 mM of the heme precursor delta-aminolevulinic acid was added. The temperature was decreased to 26°C and the cultures were incubated with shaking at 190 rpm for 40 hours. The cells were collected at 6,690g at 4C for 15 min and resuspended in 100 mM Tris base (pH 7.6), 500 mM sucrose, and 0.5 mM EDTA (15 ml buffer per g cell mass). To generate spheroplasts, 0.6 mg/ml lysozyme was added under gentle mixing and incubated with stirring for 45 min at 4°C. The mixture was then diluted 1:1 with ice cold ddH₂O and stirred for another 15 min at 4°C. Spheroplasts were collected at 10,000g at 4C for 10 min and gently mixed with lysis buffer (100 mM potassium phosphate buffer (pH 7.6), 20% (v/v) glycerol, 6 mM magnesium acetate tetrahydrate, and 0.1 mM dithiothreitol (DTT)). The spheroplasts were flash frozen and stored at -80°C until further use. To initiate CYP4F11 protein purification, spheroplasts were thawed in a water bath at room temperature. They were homogenized with a dounce homogenizer, resuspended in up to 200 ml lysis buffer, and supplemented with 1.0 ml HALT protease inhibitor.
mix (Thermo Fisher). The suspension was stirred for 15 min and sonicated for 10 min at an amplitude of 13% in 30 sec intervals. The lysed cells were centrifuged at 10,000g at 4°C for 20 min. Supernatants were carefully collected and then centrifuged at 180,000g at 4°C for 65 min to collect the *E. coli* membrane fraction. The membrane fractions were then diluted in 150 ml extraction buffer (20 mM potassium phosphate buffer (pH 7.4), 20% (v/v) glycerol, 1% (w/v) CHAPS, 1% (v/v) Tween 20, 4 mM histidine, 0.1 mM DTT, 0.1 mM PMSF) and stirred for 1.5 h. The suspension was then centrifuged at 100,000g for 30 min at 4°C. The supernatant was collected and NaCl was added to a final concentration of 500 mM. For immobilized metal affinity chromatography (IMAC), a 5 ml Histrap FF column (Cytiva) was equilibrated with 5 CV (column volumes) extraction buffer and the supernatant was loaded with a flow rate of 2 ml/min. The column was then washed with 10 CV of extraction buffer with 500 mM NaCl, and then with 10 CV extraction buffer with a decreased NaCl concentration of 100 mM (wash buffer). The protein was eluted with 10 CV elution buffer (20 mM potassium phosphate buffer (pH 8.0), 20% (v/v) glycerol, 1% (w/v) CHAPS, 1% (v/v) Tween 20, 160 mM histidine, 0.1 mM DTT, 0.1 mM PMSF) and fractions with high Soret absorbance at 420 nm vs 280 nm total protein absorbance were collected. For anion exchange chromatography, a 5 ml HiTrap Q HP column (Cytiva) was equilibrated with binding buffer (20 mM potassium phosphate buffer (pH 8.0), 20% (v/v) glycerol, 1% (w/v) CHAPS, 0.1 mM DTT, 0.1 mM PMSF). The eluted IMAC fractions were diluted 1:10 with binding buffer to decrease the salt concentration. The diluted protein was loaded on the column with a flow rate of 2 ml/min and washed with 10 CV binding buffer. CYP4F11 was then eluted with elution buffer (50 mM potassium phosphate buffer (pH 7.4), 20% (v/v) glycerol, 1% (w/v) CHAPS, 0.1 mM DTT, 0.1 mM PMSF) within a 15 CV gradient to 1 mM NaCl. Eluted protein was then exchanged into storage buffer (100 mM potassium phosphate buffer (pH 7.4), 20% (v/v) glycerol, 0.1 mM EDTA) with a 50 kDa centrifugal device (Amicon). Protein purity was
assessed by SDS-PAGE and the ratio between the Soret peak absorbance at 418 nm vs. total protein absorbance at 280 nm. The resulting Rz (Reinheitszahl) was usually around 0.7.

Recombinant human CPR was expressed and purified as described before (Sandee and Miller, 2011; Schiffer et al., 2016).

**Determination of the steady-state kinetic parameters**

To determine the kinetic parameters of CYP4F11 conversion of arachidonic acid to 20-HETE in steady-state conditions, enzymatic activity assays were performed. For this, 1 μM human CYP4F11 (concentration calculated from the CO difference spectrum) and 4 μM human CPR were reconstituted in 100 mM potassium phosphate buffer (pH 7.4) containing 100 μM DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine). Prior to use the buffer was sonicated for 5-10 min so that DLPC micelles could form. Then, arachidonic acid (dissolved in ethanol absolute) was added in a concentration range from 10-200 μM and the reaction mixture was incubated at 37°C for 3 min. The total reaction volume was 500 μl. Then, the reaction was started with 1 mM NADPH and allowed to proceed for 10 min shaking at 800 rpm. The reaction was stopped with 1 ml ethyl acetate and fatty acids were extracted twice. The extracted fatty acids were dried under vacuum and dissolved in 100% acetonitrile with 0.1% acetic acid. Using a Shimadzu Nexera HPLC-40D system, 25 μl were injected on a reverse-phase HPLC column (Restek™ Roc™ C18 HPLC Column, Particle Size: 5 μm, 150 x 4.6 mm) using an acetonitrile–water gradient (Phase A: 10% (v/v) acetonitrile/0.1% acetic acid, Phase B: 100% acetonitrile/0.1% acetic acid) as follows: 0 to 10 min 60% B (step), 11 to 25 min 80% B (step), 26 to 33 min 60% B (step), all at 40 °C and a flow rate of 0.75 ml/min. Fatty acids were detected by UV absorbance at 190 nm and the substrate and product were identified by retention times of external standards chromatographed at the beginning and ends of the experiments with a
retention time of arachidonic acid at 24 min and 20-HETE at 9 min. For kinetic analysis, the resulting 20-HETE formation [pmol]/pmol CYP4F11 per min was plotted against the respective arachidonic acid concentration resulting in a hyperbolic curve. The hyperbolic curve was fitted to a Michaelis-Menten non-linear regression model using GraphPad Prism.

**Ligand binding assays**

Spectral ligand binding assays were conducted to determine the affinity of CYP4F11 to the substrate arachidonic acid and the inhibitor HET0016. For this, 1 μM recombinant human CYP4F11 (concentration calculated from Soret absorbance) was dissolved in 1 ml 100 mM potassium phosphate buffer (pH 7.4) with the addition of 20% (v/v) glycerol for HET0016 analysis. Arachidonic acid (dissolved in ethanol absolute) was titrated to the enzyme solution in a concentration range of 10-50 μM and HET0016 (dissolved in DMSO) in a concentration range of 0.0098-2.08 μM. After each ligand addition, the solution was incubated for 8 min at room temperature to reach steady-state conditions. Then, a spectrum was recorded from 350-500 min in difference mode. The resulting change in absorbance between maximum and minimum was then plotted against the respective ligand concentration and analyzed with GraphPad Prism with a one-site specific binding fitting.

**Determination of the half-maximal inhibitory concentration with recombinant enzyme**

To determine the half-maximal inhibitory concentration ($IC_{50}$) of HET0016 on CYP4F11 conversion of arachidonic acid to 20-HETE, enzymatic activity assays were performed in presence of increasing HET0016 amounts. For this, 1 μM human CYP4F11 (concentration calculated from the CO difference spectrum) and 4 μM human CPR were reconstituted in 100 mM potassium phosphate buffer (pH 7.4) containing 100 μM DLPC. Prior to use the buffer was sonicated for 5-10 min so that DLPC micelles could form. Then, arachidonic acid (dissolved in
ethanol absolute) was added in a concentration of 75 µM which is the calculated $K_m$. Then, HET0016 (dissolved in DMSO) was added in a concentration range of 0.06-100 µM. The reaction mixture was incubated at 37°C for 3 min. The total reaction volume was 250 µl. Then, the reaction was started with 1 mM NADPH and allowed to proceed for 10 min shaking at 800 rpm. The reaction was stopped with 500 µM ethyl acetate, fatty acids were extracted twice, and then analyzed with HPLC-UV/vis analysis as described above. The percentage activity calculated from a sample without inhibitor was then plotted against the respective HET0016 concentration on a half-logarithmic scale. The data were fitted using the GraphPad Prism using the [Inhibitor] vs. response -- Variable slope (four parameters) fitting.

**Determination of the half-maximal inhibitory concentration of HET0016 on cell proliferation**

The NCI-H460 siControl and siCYP4F11 cells were seeded 24h post-transfection at the concentration of 5,000 cells/well in 96-well plate. HET0016 treatment was conducted 48 h post-transfection for 24 h at 37°C at the indicated doses (0.1 - 250 µM) with the first 3 h of HET0016 treatment without FBS. The consequences on cell proliferation were then detected by MTT Cell Proliferation Assay Kit (Abcam) following the manufacturer’s protocol. The absorbance was measured at 590 nm using a microplate reader (BioTek) and the data was normalized with GraphPad Prism to report cell viability [100%]. The percentage cell viability calculated from a sample without inhibitor was then plotted against the respective HET0016 concentration on a half-logarithmic scale. The data was then analyzed using the GraphPad Prism [Inhibitor] vs. response -- (three parameters) fitting to obtain the $IC_{50}$ values.

**Molecular Modeling**
AlphaFold (Tunyasuvunakool et al., 2021), as implemented in local ColabFold (Mirdita et al., 2022), was used to generate structures of CYP4F11. As AlphaFold structures have been found to generally be unsuitable for small molecule docking without additional refinement (Scardino et al., 2023), we generated an ensemble of structures by adjusting the size of the input multiple sequence alignment (MSA) and using multiple random seeds (Del Alamo et al., 2022). Specifically, we ran colabfold_batch with the arguments --num-seeds 5 --num-recycle 2 --num-relax 0 and --max-msa set to 16, 32, 64, 128, or 256 which resulted in a total 125 structures. In general, AlphaFold was confident in its predictions (pLDDT > 0.85) with the exception of the sequence region R275 to D315. HEME was then added to the AlphaFold structures by aligning to PDB 6C94 (CYP4B1) and extracting the HEME structure PyMOL. We then used gnina (McNutt et al., 2021) to dock HET0016 to the cavity above the HEME. Default settings were used and the bounding box was defined using the positions of HET0016 from PDB 6C94. The docked ligands were then filtered to remove poses where the iron coordinating nitrogen was farther than 3Å from the iron and from the top scoring poses three structures were selected for further refinement by molecular dynamics using Amber (Pearlman et al., 1995). Since in the AlphaFold models the C terminal helix was not interacting with the rest of the protein, to reduce simulation time the C terminal was truncated by 52 residues. Ligands and modified residues were parameterized using antechamber and the GAFF forcefield (Wang et al., 2004). Parameters for HEME were taken from literature (Shahrokh et al., 2012) and a ferric, high-spin state was used. The appropriate bonds were created between the HEME and E328, C468, and HET0016 and the system was solvated and neutralized with sodium ions using tleap. For the initial equilibration, the system was subject to two rounds of energy minimization, the first with restraints on the protein, 1ns of NTV molecular dynamics where the protein was restrained and the system was heated from 0 to 300K, and then a further 1ns of unrestrained NTP simulation at 300K and 1atm. The AMBER ff15ipq force field (Debiec et al., 2016) with TIP3P water was used due to its ability to reproduce NMR experimental observables (Koes and
Vries, 2017). For each system, three 100ns production simulations were performed. Complete simulation details are found in the Supplementary Material.

For further analysis, we chose the system that visually had the most stable secondary structure and most stable HET0016 conformation. This starting structure happened to be the most similar to PDB 6C94 in the high uncertainty region (R275 to D315). We then docked, using gnina, arachidonic acid to this structure and selected the docked pose with its omega carbon closes to the HEME iron for simulation. Simulation was performed as with HET0016, but without an explicit bond between HEME and arachidonic acid. Simulation analysis was performed using MDAnalysis (Michaud-Agrawal et al., 2011). The figures were generated with Pymol.
Results

**Transient CYP4F11 knock down attenuates cell proliferation and migration**

CYP4-mediated 20-HETE production has been shown to promote cell proliferation and migration in artificial CYP4-overexpressing cell lines. However, the role of individual 20-HETE producing CYP4 isoforms in lung cancer cell proliferation and migration has not been determined. Through analysis of the TCGA (The Cancer Genomics Atlas) database using the online tool Gepia (http://gepia.cancer-pku.cn/index.html) we found that CYP4F11 is significantly overexpressed in patients with lung squamous cell carcinoma (Figure S1, A). To examine the role of CYP4F11 in lung cancer cell proliferation and migration, we conducted a transient siRNA mediated knockdown of CYP4F11 in the lung cancer cell line NCI-H460. From Western blot analysis, we selected the siRNA with the best knockdown efficiency from three different siRNAs (supplemental Figure S1, B). Since siRNA_1 showed the highest knockdown efficiency, we used this siRNA for all subsequent work.

To examine the impact of CYP4F11 on cell proliferation, we conducted a colorimetric MTT assay and a cell counting assay to verify our observations via two independent assays. Both MTT and cell counting assay showed a significant decrease in cell proliferation of NCI-H460 siCYP4F11 cells compared to siControl cells after 24h and 48 h (Figure 2). This confirms CYP4F11 expression significantly impacts cell proliferation.

We next probed the role of CYP4F11 in cell migration which is a crucial step for cancer metastasis and determines cancer aggressiveness. Wound healing assays were conducted with NCI-H460 siCYP4F11 and siControl cells, respectively. The closing of an inserted gap was monitored after 8h, 12h, and 24 h (Figure 3). The gap closing by siControl cells was significantly increased compared to the siCYP4F11 cells indicating that a CYP4F11 knock down attenuates lung cancer cell migration *in vitro.*
The impact of CYP4F11 expression on 20-HETE production

Next, we aimed to establish a link between elevated CYP4F11 expression and 20-HETE production, since CYP4F11 is capable of metabolizing arachidonic acid to 20-HETE. The concentration of 20-HETE in NCI-H460 siControl and siCYP4F11 cells was determined via a selective ELISA immunoassay. The level of 20-HETE was drastically reduced in siCYP4F11 cells with an approximate 90% decrease of 20-HETE compared to control cells (Figure 4A).

We next examined if the addition of exogenous 20-HETE to siCYP4F11 cells rescues the cell viability. For this, a concentration of 100 nM 20-HETE was used which is 10-fold more than the reported dissociation constant required for the activation of the 20-HETE receptor GPR75 and should therefore be sufficient to observe an effect on cell viability (Garcia et al., 2017). 100 nM 20-HETE was added to siControl and siCYP4F11 cells, respectively, and an MTT assay was performed to monitor cell viability. We could observe a complete rescue of cell viability of the siCYP4F11 cells while the siControl cells were not significantly affected by 20-HETE treatment with this concentration (Figure 4B). Thus, CYP4F11-mediated 20-HETE production seems to be a key player in cell viability.

Generation of human recombinant CYP4F11

For ligand binding and inhibition assays, human recombinant CYP4F11 was successfully produced in E. coli to high purity with a typical yield of 80-100 nmol/l expression culture after IMAC and 30-70 nmol/l expression culture after the final purification step. CYP4F11 showed a typical Soret absorbance maximum at 418 nm and a maximum at 449.5 nm when reduced and complexed with CO indicating active protein (Figure S2, A and B). Notably, only little active protein could be observed after IMAC and anion exchange chromatography. Activity could be recovered after buffer exchange to the storage buffer without salt and detergents. The purity was confirmed with SDS-PAGE analysis (Figure S2, D). When reconstituted with the redox
partner CPR, the protein could convert arachidonic acid to the metabolite 20-HETE (Figure S2, C).

**CYP4F11 binding and metabolism of arachidonic acid**

We first examined metabolism and binding of the substrate arachidonic acid by recombinant human CYP4F11. To investigate the catalytic ability of CYP4F11 to convert arachidonic acid to 20-HETE, a Michaelis-Menten kinetic analysis was conducted. The resulting $K_m$ was 75.32 $\mu$M (95% CI: 51.25 - 111.8 $\mu$M) and the $k_{cat}$ was 0.21 min$^{-1}$ (95% CI: 0.18 - 0.25 min$^{-1}$) which indicates a poor turnover of arachidonic acid by CYP4F11 (Figure 5A). The obtained kinetic parameters are in good agreement with previous reported results (Tang et al., 2010).

To assess the binding affinity of arachidonic acid to CYP4F11, difference spectral ligand binding assays were performed with recombinant CYP4F11. Titration of arachidonic acid to the protein did not result in a type I shift but only in a significant decrease in absorbance between 414-421 nm with increasing ligand concentration (Figure 5B). This has been previously reported by Tang et al. (Tang et al., 2010) for arachidonic acid (20-carbon chain) and docosahexaenoic acid (22-carbon chain) but not for palmitic (16-carbon chain) and oleic acid (18-carbon chain). Addition of 100 $\mu$M arachidonic acid to CYP4F11 in full spectra mode confirmed the absence of a high spin shift of the Soret absorbance maximum to 393 nm and a decrease of Soret absorbance implicating a change in heme coordination (Figure 5B). To confirm if the heme prosthetic group was still correctly incorporated and coordinated to the axial cysteine residue upon arachidonic acid addition, we conducted CO difference spectra with and without the addition of 100 $\mu$M arachidonic acid. Addition of arachidonic acid to CYP4F11 completely converted the P450 absorbance to the P420 species which indicates inactive protein (Figure S3A). Since CYP4F11 was still active in *in vitro* metabolism assys with up to 200 $\mu$M arachidonic acid, we examined if the addition of 100 $\mu$M DLPCs to the buffer stabilizes the enzyme and repeated above described CO difference spectra in the presence of DLPCs. To our
surprise, the DLPC addition largely prevented the complete conversion of the P450 to the P420 species with arachidonic acid added and only led to a decrease in P450 absorbance (Figure S3 B). However, due to the high DLPC absorbance we were unable to confidently derive a dissociation constant for arachidonic acid and, moreover, could not detect a substrate induced high spin species under these conditions.

**CYP4F11 inhibition by HET0016**

To examine the interaction of the unselective CYP4A/F HET0016 with CYP4F11, we conducted spectral binding assays and determined the \(IC_{50}\) with recombinant human CYP4F11. When HET0016 was titrated to CYP4F11 in spectral binding assays, a type II shift could be observed, which is typical for inhibitory compounds and indicates that the terminal nitrogen of HET0016 is tightly interacting with the heme iron. The absorbance maximum was at 431-438 nm and the minimum at 411-417 nm. The calculated \(K_d\) is 332.9 nM (95% CI: 304.8 – 363.6 nM) which indicates a moderately tight binding to CYP4F11. The \(A_{\text{max}}\) was 0.02 (95% CI: 0.02 to 0.021) (Figure 6A). We then determined the half-maximal inhibitory concentration (\(IC_{50}\)) for HET0016 inhibition of 20-HETE catalysis. The \(IC_{50}\) for HET0016 was 49.5 nM (95% CI: 42 – 57 nM) which is in good accordance with the dissociation constant for HET0016 binding by CYP4F11 and indicates a strong inhibition of CYP4F11-mediated 20-HETE production (Figure 6B).

**The impact of HET0016 on cell proliferation and 20-HETE levels**

The unselective CYP4A/F inhibitor HET0016 has been a powerful tool in studying the impact of 20-HETE production on various disease states. HET0016 exhibits anti-proliferative effects on various cancer cell lines and tumors in xenograft mouse models. To investigate if HET0016 attenuates the proliferation of the lung cancer cell line NCI-H460, we determined the \(IC_{50}\) for HET0016 focusing on cell proliferation. The \(IC_{50}\) was 13.85 \(\mu\)M (95% CI: 9.83 - 19.61 \(\mu\)M),
which is a moderate inhibitory impact of HET0016 on cell proliferation. We then examined if the NCI-H460 siCYP4F11 cells were still sensitive towards HET0016 treatment assuming CYP4F11 is a target for HET0016 treatment. Here, the $IC_{50}$ was 9.84 µM (95% CI: 6.83 - 14.11 µM) showing that CYP4F11 knockdown cells still were sensitive for HET0016 treatment (Figure 7A).

Since HET0016 is known as inhibitor of 20-HETE production, we determined the 20-HETE levels for NCI-H460 cells treated with 0.1, 10, and 100 µM HET0016, respectively, and found a significantly decreased 20-HETE production compared to the control cells treated with DMSO only (Figure 7B).

**Molecular modeling reveals CYP4F11 binding of HET0016 and arachidonic acid**

To date, there is no structural information available for CYP4F11 or any other of the human CYP4 ω-hydroxylases. Thus, we conducted molecular modeling of CYP4F11 to visualize how CYP4F11 binds the substrate arachidonic acid and the inhibitor HET0016, respectively. In the CYP4F11 model with HET0016, the most consistent pose shows the hydroxylated formamidine nitrogen in close proximity to the heme iron (2.1 Å) which was set as a restrain for modeling based on the available structure of rabbit CYP4B1 with HET0016 (Jennings et al., 2018) (Figure 8 A, B). The terminal hydroxyl moiety forms two hydrogen bonds with the carbonyl oxygen of Glu-328 and Thr-332, respectively. Like in the crystal structure of CYP4B1 which we used as a blueprint for modeling, Glu-328 is covalently bound to the 5-methyl group of the heme iron. A hydrophobic interaction is observed between the methylphenyl group of HET0016 with Phe-327 in the protein I helix. The butylphenyl moiety is largely stabilized by the hydrophobic CYP4F11 active site (Phe-124, Val-395, Leu-504). Modeling of CYP4F11 with the fatty acid substrate arachidonic acid in a pose favorable for catalysis to 20-HETE revealed a highly dynamic interaction of the substrate with the enzyme active site and substrate access channel (Figure 8 C, D). While the terminal carbon of arachidonic acid remains consistently positioned over the
heme iron in a distance suitable for hydroxylation, the carboxyl group exhibits high flexibility and assumes different poses during the simulation (Figure 8 D). However, the most stable pose is formed when the carboxyl group forms hydrogen bonds with Gln-237 and the carbonyl oxygen of Val-67. This is the most abundant pose with the least r.m.s.d. (root mean square deviation) when superimposed to all frames within the simulation. Remarkably, Phe-327 assumes different rotamers within both simulations and might be crucial for keeping ligands in the active site in place.
Discussion

It is known for more than a decade that CYP4A/F-mediated 20-HETE production promotes cell proliferation and migration in cancer. Inhibition of 20-HETE production significantly decreased tumor size in lung and breast cancer xenograft mouse models. It also attenuates cell proliferation of various cancer cell lines which hint to new opportunities for cancer treatment. However, a systematic evaluation of specific 20-HETE producing CYP4F isoforms and their role in certain cancer types has, to our knowledge, only been conducted recently with a focus on CYP4F2 in lung cancer. Here, it was shown that CYP4F2-mediated 20-HETE production promotes immune suppression in tumors via interaction of 20-HETE with its receptor GPR-75 (Chen et al., 2022). Another study focuses on the artificial overexpression of the isoform CYP4A11 in lung cancer cell lines and a subsequently derived xenograft mouse models. Here, overexpression of CYP4A11 drastically increased cell migration and tumor size, while treatment with HET0016 resulted in smaller tumors. Both studies indicate a pivotal role of CYP4 \( \omega \)-hydroxylases in lung cancer progression and their high potential as drug targets. Yet, additional studies are strongly needed.

The isoform CYP4F11 is directly regulated by the transcription factor Nrf2 in lung cancer. A stable CRISPR-Cas9 mediated knockout of CYP4F11 in lung cancer cell lines resulted into less cancer cell colony formation in soft agar assays (Bar-Peled et al., 2017). However, the cause for decreased colony growth has not been further dissected. Here, we show for the first time that CYP4F11 plays a crucial role for cell proliferation and migration in the lung cancer cell line NCI-H460. To mimic potential drug action, we chose a transient siRNA-mediated gene knockout for our studies and found that both cell proliferation and migration were significantly reduced in siCYP4F11 cells (Figures 2 and 3). We could also confirm that the cellular 20-HETE levels were drastically reduced in the CYP4F11 knockdown cells indicating that indeed CYP4F11-mediated 20-HETE production triggers cell proliferation and migration (Figure 4 A). Rescue experiments with the addition of 100 nM 20-HETE to siCYP4F11 cells completely restored cell proliferation to
siControl cell proliferation levels (Figure 4B). However, addition of 100 nM 20-HETE to siControl cells did not significantly increase cell viability. We used a 20-HETE concentration of 100 nM for our studies to ensure activation of the 20-HETE receptor GPR75. The dissociation constant for 20-HETE associated receptor activation is 10 nM. Thus, we chose a 10-fold higher concentration to trigger maximal receptor activation. The use of CYP4F11 as a lung cancer drug target seems particularly attractive considering that its function in liver and kidney seems redundant to the main 20-HETE producers CYP4A11, CYP4F2, and CYP4F3B which might reduce toxicity during treatment and attenuate potential side effects. However, caution must be used since the exact function of CYP4F11 has not been completely elucidated yet.

We then generated recombinant human CYP4F11 to determine the catalysis and interaction of CYP4F11 with the substrate arachidonic acid. The resulting kinetic parameters were in good agreement with previous studies and confirms that CYP4F11-mediated 20-HETE production is overall poor (Figure 5A) (Tang et al., 2010). Other 20-HETE producing isoforms, CYP4A11, CYP4F2, and CYP4F3B, are more efficient 20-HETE producers than CYP4F11 with 10-100-fold higher $V_{\text{max}}$ values (Lasker et al., 2000; Christmas et al., 2001). However, caution must be used when comparing these kinetic parameters since the experimental parameters largely differ and many of the tested CYP4 isoforms are inserted in microsome membranes which can boost catalytic activity of membrane proteins. Arachidonic acid titration resulted in a spectral minimum around 421 nm but no clear maximum at 393 was observed which is usually the case for a substrate-induced type I shift (Figure 5B). We found that the decrease of Soret absorbance might be due to a damaging effect of arachidonic acid to the protein heme coordination which was confirmed by CO difference spectra. Upon addition of arachidonic acid, the P450 species was completely converted to an inactive P420 species which could be avoided with the addition of DLPC micelles (Figure S3). The pKa of arachidonic acid is 4.76 which is comparable to the pKa of acetic acid. It might be possible that arachidonic acid protonates the axial cysteine which leads to a loss of heme coordination. Interestingly, fatty acids with a shorter chain (C18 oleic
acid and C16 palmitic acid) led to more distinct spectral responses than arachidonic (C20) and docosahexaenoic acid (C22) in previous reports (Tang et al., 2010) suggesting a strong impact of fatty acid chain length on substrate binding. However, since CYP4F11 is indeed capable of metabolizing arachidonic acid, it is possible that only a small population of the enzyme might be able to bind the substrate with a spin state change that conventional UV/vis spectral analysis might be unable to detect due to the weakness of the signal. We conclude that CYP4F11 turnover and binding of arachidonic acid needs further investigation and propose that the traditionally used reconstitution in micelles in aqueous solution might not be an ideal system for studying CYP4F binding and catalysis of arachidonic acid and fatty acids with chain lengths greater than 20 carbons. The use of artificial membrane bilayer nanodiscs might help to reconstitute full CYP4F11 activity towards arachidonic acid. While molecular dynamics simulation indicates a robust positioning of the arachidonic \( \omega \) carbon atom when examining a pose which favors metabolism, the carboxyl moiety was highly flexible and assumed different poses within the substrate access channel (Figure 8D). Indeed, the hydrogen bonding between the carboxyl group and Val-67 and Gln-237 was the only stable interaction of arachidonic acid with CYP4F11 in addition to hydrophobic interactions in the substrate access channel. The high mobility of arachidonic acid in the active site might provide an explanation for the poor catalytic turnover.

Regardless, CYP4F11 is the only 20-HETE producing enzyme drastically overexpressed in NCI-H460 cells with 99.7 nTPM (normalized transcript expression) compared to 1.0 nTPM for CYP4F3, and no detectable expression of CYP4F2 and CYP4A11 (source: https://www.proteinatlas.org/). The higher abundance of CYP4F11 in these lung cancer cells might be sufficient to maintain a cellular 20-HETE concentration that can activate the GPR75 receptor enough to trigger cell proliferation.

We next examined the interaction of CYP4F11 with the CYP4A/F pan inhibitor HET0016. In an attempt to inhibit 20-HETE production, HET0016 was developed over two decades ago and
was a powerful tool to study the role of 20-HETE in many diseases, such as hypertension, traumatic brain injury, and cancer. The $IC_{50}$ for 20-HETE production using renal microsomes is 8.9 nM which indicates high potency (Miyata et al., 2001). A downside of HET0016 is its low selectivity for specific CYP4A/F isoforms with an $IC_{50}$ ranging from 15-100 nM for the isoforms CYP4A11, CYP4F2, and CYP4F3 (Kehl et al., 2002). We conducted spectral binding and inhibition assays to study the interaction of HET0016 with CYP4F11 as a prototypical inhibiting small molecule. Titrating HET0016 to recombinant human CYP4F11 resulted in a type II spectral shift which is typical for nitrogen-containing compounds (Figure 6 A). An X-ray protein crystal structure of rabbit CYP4B1, which is also an $\omega$-hydroxylase, reveals the terminal nitrogen of HET0016 is tightly coordinated to the heme (red circled in Figure 1 B) (Jennings et al., 2018). The dissociation constant for CYP4F11 binding of HET0016 was moderately low with 333 nM. The determined $IC_{50}$ value for HET0016 with recombinant CYP4F11 was 49.5 nM which is in the range of observed $IC_{50}$ values for other CYP4 isoforms as described above confirming poor selectivity of HET0016 for specific isoforms. Molecular dynamics simulations with CYP4F11 and HET0016 reveal the potential structural basis for the high inhibitory potency of HET0016. First, HET0016 interacts tightly with the heme iron as confirmed by spectral analysis (Figure 8 A). Second, in our molecular CYP4F11 model the terminal hydroxyl group of HET0016 stabilizes its position through hydrogen bonds with the carbonyl oxygen of Glu-328 and the Thr-332 side chain oxygen. It was reported that HET0016 only weakly inhibits CYP4 enzymes which lack the covalent link between the I helix glutamate and the heme and are not fatty acid $\omega$-hydroxylases (McDonald et al., 2017). This covalent link is required for CYP4 fatty acid $\omega$-hydroxylation. It is possible that the covalent bond positions the carbonyl oxygen into a position suitable for hydrogen bonding with the HET0016 hydroxy group and, thus, enables selective $\omega$-hydroxylation inhibition. An additional mechanism of inhibition can be explained by interaction of Thr-332 with HET0016. Thr-332 belongs to a highly conserved acid-alcohol amino acid pair
together with Asp-331 and Thr-333. This acid-alcohol pair plays an important part in the P450 catalytic cycle and is crucial for proton delivery (Hamdane et al., 2008). Thus, HET0016 blocks both substrate coordination over the heme and proton transfer needed for catalysis.

A surprising result were the similar $IC_{50}$ values for HET0016-mediated attenuation of cell proliferation for NCI-H460 siCYP4F11 and siControl cells, respectively. The $IC_{50}$ values for both cell types were not significantly different with overlapping confidence intervals and both values around 10 $\mu$M (Figure 7 A). We expected that the siCYP4F11 cells would be less sensitive for HET0016 treatment since CYP4F11 is the major 20-HETE producing enzyme in NCI-H460 cells and hence target for HET0016, but this was not the case. Analysis of 20-HETE levels of cells treated with HET0016 showed a significant reduction of 20-HETE which confirms that HET0016 indeed targets 20-HETE production (Figure 7 B). However, very high concentrations of HET0016 (10 $\mu$M) are needed to achieve half-maximal inhibition of cell proliferation, while the $IC_{50}$ of HET0016 for CYP4F11-mediated 20-HETE production with recombinant CYP4F11 is only 50 nM. An explanation for observed effects may be the poor selectivity, low stability and bioavailability of HET0016 (Borin et al., 2014; Jain et al., 2017). CYP4F11 also targets some drug metabolizing P450 enzymes with $IC_{50}$ values in a low micromolar range and their inhibition could lead to decreased cell proliferation as well (Edson and Rettie, 2013). It was previously reported that HET0016 did not affect 20-HETE levels in human glioblastoma cells and 9L gliosarcoma cells but had an inhibitory effect on cell proliferation (Guo et al., 2005; Guo et al., 2006). Thus, HET0016 might act on metabolic pathways other than the CYP4/20-HETE axis. Indeed, it has been reported that HET0016 leads to a decreased expression of cyclooxygenase-1 (COX-1) which is a potential drug target for lung cancer treatment (Jain et al., 2017; Pannunzio and Coluccia, 2018). COX-1 inhibition has similar attenuating effects on cell proliferation. Thus, more studies revealing the mechanism of action of HET0016 are required in the future.
In addition, 20-HETE might not be the only metabolite with an impact on cell proliferation, but other CYP4F11 products could play a major role in cell proliferation. CYP4F11 has been shown to catalyze the ω-hydroxylation of other fatty acids such as palmitic and oleic acid, of 3-hydroxy fatty acids, and eicosanoids, such as 15-HETE (Dhar et al., 2008; Tang et al., 2010; Skorokhod et al., 2023). While the role of other ω-hydroxylated fatty acid metabolites is not entirely known, 15-HETE has been shown to have a direct impact on lung cancer cell growth. In contrast to 20-HETE as a proliferative eicosanoid, 15-HETE has anti-proliferative properties and a lack of 15-HETE production promotes cell growth in lung cancer (Kudryavtsev et al., 2002; Li et al., 2015). It is possible that 15-HETE metabolism by CYP4F11 abolishes anti-proliferative and anti-apoptotic effects and thus, indirectly promotes cancer cell proliferation. However, more studies are needed to elucidate a potential role of CYP4F11-mediated 15-HETE metabolism. Taken together, HET0016 is not a suitable compound to study specific CYP4 isoforms in the context of human disease due to low selectivity and potential off-target effects. However, HET0016 might provide an excellent compound for future structure-based design of more specific drugs. The potential of CYP4F11 to metabolize many different fatty acids and their impact on cancer cell proliferation will be the subject of future studies.

We have shown that CYP4F11 plays a pivotal role in the cell proliferation and migration of the lung cancer cell line NCI-H460. Thus, CYP4F11 could be a new drug target for future lung cancer therapies.
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Data availability statement: The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contributions:
Participated in research design: Jia, Koes, and Brixius-Anderko
Conducted experiments: Jia, Brixius, Bocianoski, Koes, and Ray
Performed data analysis: Jia, Brixius, Koes, and Brixius-Anderko
Wrote or contributed the writing of the manuscript: Jia, Koes, and Brixius-Anderko
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changes in the bronchial airway are reflected in nasal and buccal epithelium. *BMC Genomics* **9**:259.


Footnote

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Figure legends

Figure 1:
Arachidonic acid is metabolized to the lipid mediator 20-hydroxyeicosatetraenoic acid (20-HETE) by human CYP4 enzymes via a selective ω-hydroxylation (A). The unselective CYP4 pan inhibitor HET0016 attenuates 20-HETE production (B).

Figure 2:
An siRNA-mediated transient knockdown in lung cancer cell line NCI-H460 attenuated the proliferation of NCI-H460 siCYP4F11 cells (orange line) compared to siControl cells (blue line) after day 1 and day 2 as determined by MTT assay (A) and cell counting assay (B). All data were normalized and analyzed with an unpaired Student’s t-test (n = 5-6; *, P ≤ 0.05; ***, P ≤ 0.001; ****, P ≤ 0.0001).

Figure 3:
An siRNA-mediated transient knockdown in lung cancer cell line NCI-H460 attenuated the migration ability of NCI-H460 siCYP4F11 cells (orange bars) compared to siControl cells (blue bars) as determined by wound healing assay. The gap closing was monitored after 8, 12, and 24 hours. All data were analyzed with an unpaired Student’s t-test (n = 3; **, P ≤ 0.01; ***, P ≤ 0.001).

Figure 4:
The production of the mitogenic lipid mediator 20-HETE was significantly reduced in NCI-H460 siCYP4F11 cells (orange bar) compared to the control (blue bar) as determined by 20-HETE ELISA assay (A). The addition of exogenous 20-HETE (100 nM) rescued the proliferation of siCYP4F11 cells but had no significant effect on the proliferation of siControl cells. Untreated
cells are shown in blue bars. Cells treated with 20-HETE are shown in orange bars (B). All data were analyzed with an unpaired Student’s t-test (n = 3-6; ns, P > 0.05; ****, P ≤ 0.0001).

**Figure 5:**
Kinetic analysis of CYP4F11 mediated turnover of arachidonic acid to the lipid mediator 20-HETE was performed with recombinant human CYP4F11 reconstituted with recombinant human CPR. Data are shown in technical triplicates and was fitted to the Michaelis–Menten equation to derive the kinetic parameters $K_m$ and $k_{cat}$ (A). The addition of 100 μM arachidonic acid to CYP4F11 leads to a decrease of Soret absorbance and no substrate-induced type I shift (B). These results are confirmed when performing difference spectral analysis and titrating increasing amounts of arachidonic acid (10-50 μM) to CYP4F11 (B, inset)

**Figure 6:**
Titration of the inhibitor HET0016 to recombinant human CYP4F11 induced a type II spectral shift with increasing HET0016 concentrations (A, inset). For each experiment a CYP4F11 concentration of 1 μM was used. Data shown are technical triplicates. Analysis using a one-site hyperbolic binding equation yields the fitted dissociation constant ($K_d$) and maximum absorbance change at saturation ($\Delta A_{max}$) (A). CYP4F11-mediated 20-HETE production was measured with increasing LCl699 inhibitor concentration. Data were measured in technical triplicates and fit to the dose–response inhibitor (four-parameter) equation to determine the half-maximal inhibitory concentration ($IC_{50}$).

**Figure 7:**
The half-maximal inhibitory concentration ($IC_{50}$) was determined to study the effect of the inhibitor HET0016 on cell proliferation using NCI-H460 siCYP4F11 cells (red line) and siControl cells (black line). Data were measured in replicates (n = 3-6) and fit to the dose–response
inhibitor (three-parameter) equation to determine the half-maximal inhibitory concentration ($IC_{50}$) (A). NCI-H460 cells were treated with 0.1, 10, and 100 μM HET0016 and the 20-HETE production was determined with a 20-HETE ELISA assay. All data were normalized and analyzed with an unpaired Student's t-test ($n = 2-3$; *, $P \leq 0.05$; **, $P \leq 0.01$) (B).

**Figure 8:**

Molecular modeling to obtain a CYP4F11 protein structure model in complex with the inhibitor HET0016 (A, B, magenta) and the substrate arachidonic acid (C, D, cyan). HET0016 binds to the CYP4F11 heme iron and is further coordinated with the protein I helix via hydrogen bonding. Arachidonic acid is high flexible within the active site and aligns along the hydrophobic CYP4F11 substrate access channel. Multiple possible poses for arachidonic acid are shown in grey sticks with the terminal carbon atom positioned over the heme iron.
Figure 2

(A) Cell viability %

- siControl
- siCYP4F11

Cell counting

Day 0  Day 1  Day 2

(B) Cell viability %

- siControl
- siCYP4F11

MTT assay

Day 0  Day 1  Day 2

*  ***  ****
Figure 4
Figure 5

![Graph A showing the relationship between Arachidonic acid concentration and $K_m$, with $K_m = 75.32 \, \mu M$ and $k_{cat} = 0.21 \, min^{-1}$.]

![Graph B showing absorbance spectra with and without 100 $\mu M$ AA.]

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Figure 6

A. $K_d = 332.9$ nM

$A_{max} = 0.02$

B. $IC_{50} = 49.5$ nM
Figure 7

(A) Cell viability (%) as a function of HET0016 concentration. 
- siControl
- siCYP4F11

IC_{50} = 13.85 \mu M
IC_{90} = 9.84 \mu M

(B) 20-HETE production [%] as a function of HET0016 concentration.

* * **
Figure 8