Structure Guided Chemical Modifications of Propylthiouracil Reveal Novel Small Molecule Inhibitors of Cytochrome b₅ Reductase 3 That Increase Nitric Oxide Bioavailability*

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Background: Cytochrome b₅ reductase 3 (CYB5R3) regulates nitric oxide (NO) diffusion in the artery wall.

Results: Novel CYB5R3 small molecule inhibitors were discovered that increase NO bioavailability.

Conclusion: A potent new CYB5R3 inhibitor improves vascular function.

Significance: These data provide a platform for further drug development and new tools for understanding CYB5R3 function.

NADH cytochrome b₅ reductase 3 (CYB5R3) is critical for reductive reactions such as fatty acid elongation, cholesterol biosynthesis, drug metabolism, and methemoglobin reduction. Although the physiological and metabolic importance of CYB5R3 has been established in hepatocytes and erythrocytes, emerging investigations suggest that CYB5R3 is critical for nitric oxide signaling and vascular function. However, advancement toward fully understanding CYB5R3 function has been limited due to a lack of potent small molecule inhibitors. Because of this restriction, we modeled the binding mode of propylthiouracil, a weak inhibitor of CYB5R3 (IC₅₀ ≈ 275 μM), and used it as a guide to predict thioriouracil-biased inhibitors from the set of commercially available compounds in the ZINC database. Using this approach, we validated two new potent derivatives of propylthiouracil, ZINC05626394 (IC₅₀ = 10.81 μM) and ZINC39395747 (IC₅₀ = 9.14 μM), both of which inhibit CYB5R3 activity in cultured cells. Moreover, we found that ZINC39395747 significantly increased NO bioavailability in renal vascular cells, augmented renal blood flow, and decreased systemic blood pressure in response to vasoconstrictors in spontaneously hypertensive rats. These compounds will serve as a new tool to examine the biological functions of CYB5R3 in physiology and disease and also as a platform for new drug development.

NADH cytochrome b₅ reductase 3 (CYB5R3⁵): NADH: ferri-cytochrome b₅ oxidoreductase, EC1.6.2.2) or methemoglobin reductase is a flavoprotein known for its ability to transfer electrons from the NADH domain of CYB5R3 to cytochrome b₅. In somatic cells membrane-restricted CYB5R3 regulates several biological reduction reactions including elongation and desaturation of fatty acids (1), cholesterol biosynthesis (2), and drug metabolism (3, 4). The soluble form of CYB5R3, present in erythrocytes, reduces methemoglobin (5, 6). In the human population, genetic mutations leading to deficient CYB5R3 activity causes recessive hereditary methemoglobinemia (7). Type I methemoglobinemia only affects erythrocytes, causing increased levels of methemoglobin in these cells (7), whereas Type II methemoglobinemia affects all somatic cells causing severe developmental neurological disorders (8). Recent evidence suggests that membrane-bound CYB5R3 expression and activity also contributes to metabolic homeostasis (9), stress protection (9), and nitric oxide (NO) bioavailability (10).

Nitric oxide, a naturally produced biogas, contributes to diverse biological processes and is a well known potent vasodilator (11–14). Recent work revealed both α globin and CYB5R3 expression in small artery and arteriolar endothelial cells (ECs) at the myoendothelial junction (MEJ), the anatomical location where the endothelium and vascular smooth muscle cells (VSMC) make contact (10). The expression of α globin and CYB5R3 in endothelium regulates NO diffusion to VSMC via biochemical reactions of NO with α globin and CYB5R3 (5, 6, 7). Whereas NO from endothelial nitric-oxide synthase can react with oxygen-bound ferrous heme iron (Fe²⁺) α globin, resulting in NO scavenging, ferric heme iron (Fe³⁺) α globin permits NO diffusion due to its slow and weak reaction. CYB5R3 serves as a switch to

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5 The abbreviations used are: CYB5R3, cytochrome b₅ reductase 3; EC, endothelial cell; MEJ, myoendothelial junction; VSMC, vascular smooth muscle cell; PtU, propylthiouracil; SHR, spontaneously hypertensive rat; MABP, mean arterial blood pressure; HR, heart rate; RBF renal blood flow; RVR, renal vascular resistance; AngII, angiotensin II; NE, norepinephrine; NPY, neuropeptide Y; ITC, isothermal titration calorimetry; methemoglobin.
control the heme iron redox state of \( \alpha \) globin, allowing it to modulate NO bioavailability and arterial vascular tone (10). Thus, CYB5R3 serves as an attractive biological target to increase NO bioavailability to augment microcirculatory blood flow and decrease blood pressure in the setting of cardiovascular disease.

Currently there are no potent small molecule inhibitors that block CYB5R3 activity. Previous studies demonstrated that propylthiouracil (PTU), a drug designed to treat hyperthyroidism, inhibits CYB5R3 at a concentration of \( \sim 275 \mu M \) (15). However, it is still unclear how PTU exerts its inhibitory effects at a molecular level. By understanding the mechanistic action by which PTU inhibits CYB5R3, a new class of inhibitors with good potency, selectivity, and cellular activity could emerge to study the function of these enzymes.

In this study a docked model for PTU at the NADH binding site of the protein was used to perform a structure-based pharmacophore screen of commercially available ZINC compounds (16). By implementing this approach, two new thiouracil derivatives were discovered, ZINC39395747 (IC\(_{50} \) = 9.14 \( \mu M \)) and ZINC05626394 (IC\(_{50} \) = 10.81 \( \mu M \)). Studies using derivatives of ZINC39395747 and ZINC05626394 revealed the mechanism by which thiouracil compounds block CYB5R3 activity. To examine the biological effects of ZINC39395747, vascular cell culture studies were performed showing that ZINC39395747 increases NO bioavailability. Lastly, \( \text{in vivo} \) inhibition of CYB5R3 with ZINC39395747 resulted in augmented renal blood flow and decreased blood pressure in spontaneously hypertensive rats infused with vasoconstrictors. Together, these results uncover novel compounds that inhibit CYB5R3 activity and can increase NO bioavailability in vascular cells. These compounds can serve as a basis for further drug development to improve blood flow and possibly for treatment of hypertension and cardiovascular disease. Moreover, these compounds will be a valuable tool in understanding the diverse biological roles of CYB5R3 in health and disease.

**Experimental Procedures**

*Docked Model*—This model was refined by analysis of the CYB5R3 crystal structure in the RCSB Protein Data Bank (PDB ID 1UMK). The crystal structure of CYB5R3 and a close homolog (PDB ID 1QFY) that contains the cofactors flavin adenine dinucleotide (FAD) and NADH were used. Superposition of PTU on the NADH ring followed by energy minimization with Smina (17), a fork of AutoDock Vina (18) that is customized to better support scoring function development and high performance energy minimization, led to the proposed position of PTU in the NADH pocket of CYB5R3.

*Small Molecule Selection*—After establishing this model, a thiouracil-based pharmacophore screening of the commercially available compounds in the ZINC database was performed (19). The receptor structures were prepared using a script provided by the open source software AutoDock to set up the receptor structure for docking. The best ranking molecules were reviewed and chosen based on chemical diversity and potential interactions. For the follow-up assay, a selection was made of compounds that were chemically similar to the most potent inhibitors, ZINC05626394 and ZINC39395747. These compounds were identified by performing a 70% similarity search of the ZINC database. The compounds were selected based on the desired substituents for a detailed structure and activity relationship.

*Modeled Chemicals and Purity*—All modeled chemicals with ZINC numbers, MolPort ID, supplier information, and catalogue numbers are listed in Table 1. We determined the purity of the small molecule inhibitors via NMR analysis. To do so, \( \sim 5 \) mg of ZINC05626394 and ZINC39395747 was initially dissolved in DMSO followed by dilution into CDCl\(_3\). \(^1H\) NMR spectra were acquired using a Bruker Avance III 400 MHz with a \(^13C\),\(^1H\) DUL BBO observe probe (Karlsruhe, Germany). The ZINC05626394 and ZINC 39446575 NMR spectra were provided by the manufacturer. All the compounds were pure (data not shown).

*Cloning, Expression, and Purification of Human Cytochrome b\(_5\) Reductase 3 and Cytochrome b\(_6\)*—Purified proteins were prepared as previously described in Sparacino-Watkins *et al.* (20). Briefly, recombinant human CYB5R3 and CYB5B were isolated from *Escherichia coli* SoluBL21 cells (Genlantis) transformed with the CYB5R3 gene cloned into the pET28a plasmid and...
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CYB5B cloned into a pET11a plasmid. A Hisx tag on the N terminus of CYB5R3 replaces the mitochondrial leader sequence. The C-terminal mitochondrial leader sequence of CYB5B was also removed, but no affinity tag was included. Protein production and purification was carried out as previously described (21, 22). Concentrations of CYB5R3 and CYB5B were measured with UV-visible spectroscopy (Cary 50 spectrophotometer) using the previously published extinction coefficients for CYB5R3 (ε462 nm = 10.4 mm−1 cm−1) and CYB5B (ε414 nm = 117 mm−1 cm−1) (21). Chromatographic separation was conducted with an Äkta-Purifier FPLC (GE Healthcare) running Unicorn software Version 5.1. Metal affinity chromatography resin, Ni-NTA superflow (Qiagen), was packed into a XK 26/20 column (GE Healthcare) to isolate CYB5R3. CYB5B was isolated using anion exchange (DE32, Whatman) followed by a gel filtration column (GE Healthcare). Protein identity was confirmed with liquid chromatography and tandem mass spectrometry (LC-MS/MS, University of Pittsburgh Genome and Protein Core Facilities).

Purified CYB5R3 Activity Assay—The activity of purified CYB5R3 was assayed utilizing the NADH-ferricyanide reduction reaction. The reduction rate at 420 nm by CYB5R3 was assessed via spectrophotometric measurements according to Strittmatter and Velick (23). The assay mixture contained 0.1 M potassium phosphate buffer, pH 7.5, containing 10 mM potassium ferricyanide, 5 mM NADH, and 90 mM concentrations of purified CYB5R3 in a final volume of 200 µl. The reaction was started by the addition of the cofactor NADH, and reduction of ferricyanide was followed for 2 min by recording the absorbance decrease at 420 nm using a Cary 50 spectrophotometer in small glass cuvettes with a 0.2-cm path length. Because NADH has partial reduction power independent of CYB5R3, the reaction rate was corrected by subtracting the reaction rate of ferricyanide in the absence of enzyme. The enzyme activity was calculated using the extinction coefficient of 1.02 mm−1 cm−1 for the difference in absorbance between the reduced and oxidized form of ferricyanide.

To test the inhibitory effect of each CYB5R3 small molecule inhibitor, the compounds were preincubated with CYB5R3 at 37 °C for 60 min followed by measurements of NADH-ferricyanide reductase activity as described above. For primary screening, 500 µM concentrations of each compound were used, and compounds that gave 100% inhibition of CYB5R3 were selected for a secondary screen where 50 µM concentrations of each compound were tested. Finally, compounds that gave 100% inhibition at 50 µM were evaluated at different concentrations, and IC50 values were calculated using GraphPad Prism software by linear fit.

Analysis of Small Molecule Inhibitor Binding with CYB5R3 by Isothermal titration Calorimetry (ITC)—ITC was used to analyze the binding of the small molecule inhibitors with CYB5R3. An ITC instrument made by TA Instruments (Lindon, UT) was used that has a reference cell volume of 990 µl, a sample cell volume of 992 µl, and an injector syringe volume of 100 µl. Calorimetric titration of CYB5R3 was made with 0.4% DMSO in a 100 mM potassium phosphate buffer, pH 7.4, in the reference cell. The temperature was set to 25 °C, and 1 ml of 10 µM CYB5R3 was loaded in the sample cell and titrated with 20 continuous injections of 4.95 µl of 200 µM PTU, ZINC05626394, and ZINC39395747 to determine the binding constant. The injections were made at 5-min intervals, and the binding constant was determined by calculating the change of heat (dH) in subsequent injections using the Nanoanalyze software provided with the ITC instrument.

In Vitro Assay to Determine Temporal Inhibition of ZINC39395747 on CYB5R3 Activity—To determine the kinetics of ZINC39395747 inhibition on CYB5R3 activity, a 180 nM concentration of recombinant CYB5R3 was incubated with 18.28 µM ZINC39395747 at 37 °C for 2 min to 24 h, and subsequent activity of CYB5R3 was measured by ferricyanide assay as previously described above with the addition of 10 mM potassium ferricyanide and 5 mM NADH.

In Vitro Assay to Analyze Binding Strength of ZINC39395747 with CYB5R3—To analyze binding affinity of ZINC39395747 with CYB5R3, a 180 nM concentration of recombinant CYB5R3 was incubated with 18.28 µM ZINC39395747 in 100 mM potassium phosphate buffer, pH 7.5, at 37 °C for 1 h. Then unbound drugs were removed by using Millipore size exclusion column with cutoffs of M, 3000. After 2 washes, the protein volume was adjusted with the same buffer and divided into 2 fractions. One fraction was immediately used to determine CYB5R3 activity via the ferricyanide assay as described above. The second fraction was incubated at 37 °C for 12 h before assessing CYB5R3 activity.

Cellular CYB5R3 Activity Assay—Cellular CYB5R3 activity was determined according to the method by Siendones et al. with modifications (9). In brief, human embryonic kidney cells and isolated renal ECs as previously described (24) were plated in 6-well plates and treated with 21.86 µM ZINC05626394 and 18.28 µM ZINC39395747 for 24 h. After treatment, cells were washed 2 times with PBS, trypsinized, and centrifuged at 1000 × g for 5 min to pellet cells. The pellet was resuspended in 500 µl of buffer A (130 mM Tris-HCl, pH 7.6, 0.1 mM DTT, and 1 µM protease inhibitor mixture). Cells were homogenized with a micro pestle and centrifuged at 2000 × g for 10 min at 4 °C followed by a collection of supernatant. To test for CYB5R3 activity, 30 µg of total lysate was incubated with 1 mM Tris-HCl, pH 7.6, 0.5 mM EDTA, 2 mM potassium ferricyanide, and 0.25 mM NADH for 2 min at 25 °C. Absorbance changes at 420 nm were measured to determine ferricyanide reduction by CYB5R3.

CYB5R3 Knockdown—To confirm specificity of the cellular CYB5R3 activity assay, CYB5R3 stable knockdown HEK cells were generated with lentivirus. The shRNA construct was purchased from Sigma (TRCN0000236407). The ViralPower Lentiviral Expression System (Invitrogen) was used to generate lentivirus with the following modifications. For transfection, two mixtures of reagents were prepared. Mix 1 contained 5.6 µg of pLP1, 2.4 µg of pLP2, 4 µg of pLP/VSVG, and 10 µg of pLKO.1-CYB5R3 shRNA in 0.5 ml of Opti-MEM medium. Mix 2 contained 50 µl of Lipofectamine 2000 in 0.5 ml of Opti-MEM, which was incubated for 5 min at room temperature. Next, the two mixtures were combined for 20 min at room temperature and then added dropwise to HEK 293FT cells grown to 90–95% confluency in a 10-cm2 dish. After 5 h, the media were replaced with Freestyle 293 plus 5% penicillin/streptomycin. After 72 h
of culture, viral supernatant was collected and centrifugated at 1000 rpm for 5 min to pellet the cell debris. The viral supernatant was then concentrated using an Amicon Ultra-15 filter unit with a 100-kDa molecular weight cutoff. For lentivirus transduction, 20 μl of concentrated virus was added to HEK 293 cells at 50% confluency in a 35-mm² dish with 10 μg/ml Polybrene. Twenty-four hours after transduction, cells were selected for 10 days with 1 μg/ml of puromycin to generate stable CYB5R3 knockdown cells. Knockdown was confirmed via Western blot analysis using CYB5R3 (Protein Tech) and tubulin (Sigma) primary antibodies followed by Licor secondary antibody detection. Images were obtained using a Licor Odyssey and quantified using Licor image analysis software.

Met a Globin Reduction Assay—To confirm the efficacy of the small molecule inhibitors on CYB5R3 activity, purified met a globin chains were isolated as previously described (10). To begin, α globin chains were incubated with 100 nm CYB5B and 100 nm CYB5R3 in PBS, pH 7.4, followed by pretreatment with 21.86 μM ZINC39395747, 18.28 μM ZINC39395747, or DMSO. Next, NADH was added to a final concentration of 250 μM and met α globin reduction was monitored by scanning UV absorbance between 400 and 700 nm at room temperature for 5 min. Differences were determined by plotting the percentage of optical density change at 577 nm and 630 nm over time.

Methemoglobin Formation Assay in Mouse RBCs—To analyze methemoglobin formation in the presence of ZINC39395747, fresh blood was drawn from the left ventricle of the heart using a heparin-coated needle and syringe from wild type C57Bl/6 mice (Taconic Farms) according to University of Pittsburgh Animal Care and Use committee guidelines. Twenty-four hours after transduction, cells were selected for 10 days with 1 μg/ml of puromycin to generate stable CYB5R3 knockdown cells. Knockdown was confirmed via Western blot analysis using CYB5R3 (Protein Tech) and tubulin (Sigma) primary antibodies followed by Licor secondary antibody detection. Images were obtained using a Licor Odyssey and quantified using Licor image analysis software.

Results
Rational-based Drug Screen Identifies New Inhibitors of CYB5R3—CYB5R3 contains a FAD as a non-covalently bound cofactor. FAD facilitates electron transfer processes via its isoalloxazine ring that can accept electrons at different positions. Oxidized FAD-bound CYB5R3 uses two electrons from NADH through hydride transfer to fully reduce FAD to transfer single electrons to acceptors such as methemoglobin. The docked model intervenes at the initiation of the process where NADH acts as the initial electron donor. As shown in Fig. 1a, CYB5R3 consists of two domains with the bound FAD cofactor (orange), a FAD binding domain (Thr–30–Ser–145, blue), and a NADH binding domain (Ser–173–Phe–300, red). These domains are connected by a linker (hinge) sequence at the bottom (Gly–146–Lys–172, purple), which allow the domains to move away and toward each other (PDB ID 1UMK) (29). There are no crystal structures of human CYB5R3 that contain NADH; therefore, the NADH binding
mode was obtained by analysis and alignment with non-NADH-bound human CYB5R3 and computational guided NADH superpositioning (29, 30). This method provided a suggested binding mode for NADH at its corresponding binding site in CYB5R3 (Fig. 1b). Docking of PTU in the NADH site using Smina led to the proposed binding model for thiouracil (Fig. 1c). We rationalized that thiouracil binds to the NADH site of the protein where it stacks with the isoalloxazine rings, which is similar to the stacking arrangements of NADH. This binding mode allows for four specific interactions between PTU and the binding pocket; O11 accepts a hydrogen from Thr-181 side chain OH, N3 donates a hydrogen to Thr-184 side chain OH, S7 accepts hydrogen from terminal OH of Phe-300, and N1 donates hydrogen to the terminal carbonyl of Phe-300 (Fig. 1c).

After establishing a docking model, a thiouracil-biased pharmacophore screening of commercially available compounds with modifications to the side chains or the carbon tail of the uracil ring in the ZINC database was performed using the ZincPharmer server (Fig. 1d and e) (30). The resulting compounds were energy-minimized and scored against the receptors 1UMK (29) and 1QFY (30), and the best ranking molecules were reviewed and chosen based on chemical diversity and predicted interactions. For the initial screen, 13 commercially available modified PTU derivatives were found and tested for CYB5R3 inhibition (Fig. 1f). In the primary screen, these compounds were used at a concentration

![Flow chart representing the approach taken to identify new small molecule inhibitors for CYB5R3.](image_url)
of 500 μM. This revealed four compounds, ZINC05626394, ZINC31773411, ZINC39395747 and ZINC13957379, which exhibited 100% inhibition, similar to PTU (Fig. 2a). Next, we executed a secondary screen for the four compounds that provided 100% inhibition in the primary screen, dropping the concentration from 500 μM to 50 μM (Fig. 2b). Two compounds, ZINC05626394 and ZINC39395747, exhibited 100% inhibition. These two inhibitors were tested at lower concentrations to determine the IC₅₀ values (Fig. 2c). It should be noted that ZINC05626394 and ZINC39395747 have IC₅₀ values of 10.81 μM and 9.14 μM, respectively, a major improvement over the PTU IC₅₀ value of 279.40 μM.

We next analyzed PTU, ZINC05626394, and ZINC39395747 with ITCs to determine independent $K_r$, reaction stoichiometry,
and binding of each inhibitor with CYB5R3. Results demonstrate that the binding constant for PTU is 25.04 μM, whereas the binding constants for ZINC39395747 and ZINC05626394 are 1.11 μM and 6.83 μM, respectively (Fig. 2d). Next, we performed a time course to measure the ability of ZINC39395747 to inhibit CYB5R3 and found that t_{1/2} = 26 min (Fig. 2e). We also observed that after removal of unbound ZINC39395747, the inhibition was 88.03 ± 3.50 and 89.14 ± 2.64 at 0 and 12 h incubation, respectively.

To further analyze the structure activity relationship, we searched the ZINC database for commercially available compounds that had 70% chemical similarity to ZINC39395747 and ZINC05626394. We found six similar compounds for ZINC05626394 and 1 compound for ZINC39395747 (Fig. 3, a and b) (16). After a primary screen with 500 μM and a secondary screen with 50 μM, we found that ZINC05626394 had an IC_{50} value of 15.1 μM (3, c and d).

CYB5R3 Inhibitors Block Cellular Activity and Metglobin Reduction in Vitro—To determine if the most potent compounds, ZINC05626394 and ZINC39395747, exert inhibitory activity in HEK and renal ECs, we subjected the cells to an in vitro CYB5R3 activity assay. First, we tested the specificity of the assay using stable HEK CYB5R3 knockdown cells generated using lentivirus. Knockdown of CYB5R3 protein was 75% (Fig. 4a), which resulted in a 65% loss in activity (Fig. 4b). Using the same assay, HEKs were treated for 24 h with ZINC05626394 and ZINC39395747, which showed >80% inhibition of cellular CYB5R3 activity (Fig. 4c). Renal ECs treated with ZINC05626394 and ZINC39395747 for 24 h showed >75% inhibition (Fig. 4d). We observed no change in CYB5R3 protein expression in renal endothelial cells following CYB5R3 activity inhibition (Fig. 4e). To analyze direct inhibition of CYB5R3 inhibitors on met α globin reduction, purified met α globin was generated. Our results show that the rate of α globin reduction by CYB5R3 was significantly inhibited with ZINC05626394 and ZINC39395747 (Fig. 4f).

CYB5R3 Inhibition Does Not Form MetHb in RBCs Acutely—Loss of CYB5R3 activity is reported to associate with methemoglobinemia (31, 32). Therefore, we tested if ZINC39395747 causes methemoglobin formation in intact live red blood cells. Our results demonstrated that 24-h post ZINC39395747 treatment caused no methemoglobin formation (Fig. 4g). However, after 36 and 48 h we noted elevated levels of methemoglobin indicated by an increase in absorbance at the 630 nm (Fig. 4, g and h).

CYB5R3 Inhibition Increases NO Bioavailability—Previous work demonstrated that reduced CYB5R3 activity leads to α globin heme iron oxidation resulting in augmented NO diffusion from endothelium to VSMC (10). We conducted a similar study using renal ECs and pre-glomerular VSMCs. Results in Fig. 5a show expression levels of CYB5R3 in EC, MEJ, and VSMC fractions and α globin enrichment at the MEJ consistent with our previous work (10). Because NO binds to and activates soluble guanylate cyclase leading to increased cGMP in vascular smooth muscle, we tested the effects of ZINC39395747 on NO diffusion using this co-culture system. Co-cultures were

![List of ZINC compounds with modified pyrimidine rings and their inhibitory effect. a shows the derivatives of ZINC05626394, and b shows the ZINC39395747 derivative with modified pyrimidine rings. c illustrates the primary screen using recombinant human CYB5R3 incubated with each compound (500 μM) followed by activity measurements with an NADH-ferricyanide reductase assay. d represents a secondary screen with 50 μM concentrations. All error bars represent the S.E.](image-url)
pretreated with ZINC39395747 followed by the addition of the NO donor DEA NONOate to the EC side of the co-culture. After 30 min, VSMC lysates were collected and measured for cGMP, which showed a significant increase in cGMP, indicating that blockade of CYB5R3 with ZINC39395747 increased NO diffusion (Fig. 5b).

**CYB5R3 Inhibition Improves Renal Blood Flow following Vasoconstriction**—Our previous data demonstrated that genetic knockdown of CYB5R3 increases NO diffusion from endothelium to smooth muscle, which attenuates vasoconstriction (10). Consistent with these data, other reports showed that inhibition of nitric-oxide synthase activity in vivo augments vasoconstrictor effects of AngII and NE causing decreased renal blood flow and increased systemic blood pressure (33), whereas neuropeptide Y1–36 infusion was reported to have little effect (34). To assess whether blockade of CYB5R3 alters blood pressure and renal hemodynamics in response to vasoconstrictors, we infused ZINC39395747 (1 mg/kg) through the jugular vein in spontaneously hypertensive rats. Pre and post injection measurements of MABP (192.91 ± 5.7 versus 174.67 ± 4.7 mm Hg; *, p < 0.05), RBF (5.975 ± 0.135 versus 6.787 ± 0.413), and heart rate (362.9 ± 10.05 versus 354.8 ± 9.44 beats per min) were obtained after ZINC39395747 administration. Next, measurements of MABP, RBF, and RVR were recorded in response to AngII, NE, and NPY. Significant differences in MABP, RBF, and RVR were observed after a dose response to AngII and NE (Fig. 6, a and b). However, NPY infusion did not result in significant changes in MABP, RBF, and RVR with ZINC39395747 (Fig. 6c). To ensure that the lack of NPY effect was not due to drug metabolism, we performed an additional study whereby a NPY dose response was immediately performed after ZINC39395747 administration. We observed no significant effect on MABP, RBF, and RVR (data not shown), which is consistent with the effects shown in Fig. 6. After the completion of agonist infusion, we collected blood and measured methemoglobin formation and no difference between control and ZINC39395747-treated animals (data not shown).

**FIGURE 4. Effects of ZINC05626394 and ZINC39395747 on cellular CYB5R3 activity.** a, CYB5R3 knockdown efficiency in HEK cells using shRNA. b, CYB5R3 activity inhibition in HEK CYB5R3 knockdown cells determined by ferricyanide reduction assay. CYB5R3 activity inhibition by ZINC39395747 and ZINC05626394 in HEK cells (c) and rat renal endothelial cells (d) was determined by ferricyanide reduction assay. e, CYB5R3 protein levels in rat renal endothelial cells treated with ZINC39395747 and ZINC05626394. f, reduction rate of met α globin by CYB5R3 in the presence of ZINC39395747 and ZINC05626394. g, met α globin formation in isolated mouse RBCs treated with ZINC39395747 for 36–48 h. AU, absorption units. h, a representative image of met α globin formed in RBCs treated with ZINC39395747 or vehicle. * indicates significant difference of p < 0.05. All error bars represent S.E.
Discussion

It is well established that augmented levels of NO cause vasodilation resulting in improved blood flow and lower blood pressure. In the setting of cardiovascular disease, decreased NO levels are a hallmark of arterial vascular dysfunction, leading to increased vasoconstriction and hypertension. Recent evidence has demonstrated that loss of endothelial cell CYB5R3 increases NO bioavailability in the vascular wall by regulating the redox state of α globin (10). Because loss of CYB5R3 expression increases NO signaling, it is possible that inhibition of CYB5R3 could serve as an attractive target to reduce vasoconstriction. For this reason we set forth to identify new small molecule inhibitors of CYB5R3. By taking advantage of known information about PTU, a weak inhibitor of CYB5R3, and the crystal structure of CYB5R3, we have for the first time revealed the structural basis of inhibition of CYB5R3 by PTU. Using this insight, we utilized a rational approach to develop more potent chemical probes to study the function of this enzyme in vivo, a strategy that can be generalized to similar targets. Specifically, through superpositioning of PTU in the NADH binding pocket of CYB5R3 followed by a pharmacophore screen of commercially available PTU derivatives, we successfully identified two compounds (ZINC05626394 and ZINC39395747) that decrease the IC₅₀ by 30-fold compared with PTU. Furthermore, through selective replacement of specific molecules in the pyrimidine or phenyl ring, we gained important mechanistic insight into the mode of action by which PTU and these compounds exert their inhibitory action. To do so, we performed a screen to identify compounds that were chemically similar by using the 70% similarity search in the ZINC database of commercially available compounds (16). Two series of compounds were selected that established a structure and activity relationship around the pyrimidine and phenyl ring. The first series consists of two ZINC05626394-like compounds that substitute the sulfur on the pyrimidine ring for a carbon (ZINC05774833) or an oxygen (ZINC17744101) (Fig. 3a). The second ZINC39395747-like compound substitutes the sulfur for an oxygen in the pyrimidine ring (Fig. 3b). Interestingly, the replacement of sulfur by oxygen in ZINC17744101 showed 100% inhibition using 500 μM and 50% inhibition at 50 μM, whereas PTU showed little inhibition at the 50 μM concentration (Fig. 3, c and d). The reveal a new class of CYB5R3 inhibitors that can be used both in cells and in vivo to advance our understanding of the role of CYB5R3 in NO signaling and, equally important, presents a starting point for the design of future drugs to enhance NO bioavailability.
S-CH₃ modification in ZINC05774833 and ZINC 39446575 had only partial inhibitory effects even at the 500 μM concentration (Fig. 3c).

The ability of PTU to treat hyperthyroidism may depend on its capacity to function as a thiol, which could also be true for the inhibition of CYB5R3 activity. Given the similarity of our compounds to PTU, we cannot rule out the possibility that similar effects could be taking place in our structure and activity relationships. Moreover, it is conceivable that the inhibitors favor double-bonded sulfur as inhibition is
achieved via this covalent bond between Cys-273 and the inhibitor.

We then extended our study to physiological and pathological models. First, we determined if ZINC05626394 and ZINC39395747 inhibited CYB5R3 activity in HEK and rat renal ECs. We observed >75% inhibition of CYB5R3, suggesting that the compounds are cell-permeable and effectively block CYB5R3 activity (Fig. 4, c and d). Because CYB5R3 reduces oxidized α globin, we then tested the ability of ZINC05626394 and ZINC39395747 to inhibit this process. Results shown in Fig. 4f demonstrate significant inhibition of oxidized α globin reduction with ZINC05626394 and ZINC39395747.

It has been reported that reduced activity of CYB5R3 is associated with methemoglobinemia (31, 32). Interestingly, our results suggest that inhibition of CYB5R3 activity by ZINC39395747 in isolated RBCs does not cause methemoglobin formation in 24 h (Fig. 4g). However, our results suggest accumulation of methemoglobin occurs around 36 h (Fig. 4h). In a SHR rat model, there was no significant level of acute methemoglobin formation after infusion of ZINC39395747. The aggregate of these results suggests that RBC methemoglobin formation and loss of oxygen binding and distribution are likely not a contributing factor to the hemodynamic effects we observed in SHR. It is conceivable however, that long term exposure to ZINC39395747 could result in methemoglobin formation. However, this effect will likely depend on the rate of metabolism of ZINC39395747, in which more detailed studies are required.

Previous reports showed that loss of nitric-oxide synthase activity exacerbates renal vasoconstriction after acute infusion with AngII or NE (33). Consistent with this study, our work has demonstrated that CYB5R3 in ECs regulates the redox state of α globin to control nitric oxide diffusion to vascular smooth muscle during vasoconstriction (10). Therefore, using a SHR model, we examined the acute effects of CYB5R3 inhibition on MABP, RBF, and RVR. After 1-h post ZINC39395747 infusion, we observed a significant decrease in MABP consistent with our previous finding that CYB5R3 increases NO bioavailability thereby lowering peripheral vascular resistance and thus blood pressure. We then extended our studies to determine the effects of CYB5R3 inhibition after AngII and NE, and NPY infusion. Consistent with previous reports demonstrating that NO serves to modulate vasoconstriction in response to AngII and NE, we found that ZINC39395747 significantly inhibited the increase in MABP, RBF, and RVR in SHRs. However, this response was not observed after NPY infusion. These results are consistent with a previous report from canine coronary arteries showing that NPY-induced vasoconstriction does not stimulate the release of NO (34). Although there was no effect of NPY on renal MABP, RBF, and RVR, the significant effect of AngII and NE provides evidence that blockade of CYB5R3 may be critical for regulation of α globin heme iron redox status to control NO diffusion. Although these data are consistent with our previous report, implicating that loss of CYB5R3 protein or inhibition of CYB5R3 with PTU treatment increases NO signaling (10), we cannot rule out other potential mechanisms that could regulate this response. For example it is known that cyto-globin is abundantly expressed in vascular smooth muscle, where it can regulate NO scavenging through a similar mechanism as α globin (10, 35). It is possible CYB5R3 in vascular smooth muscle could regulate the heme iron redox state of cytoglobin similar to α globin. However, more studies are required to verify this potential mechanism. From a therapeutic perspective, we envision that reducing CYB5R3 activity rather than complete blockade may be an ideal approach to increase NO bioavailability. This is particularly important as NO-stimulated soluble guanylate cyclase activity amplifies cGMP production in vascular smooth muscle leading to vasodilation. It is estimated that a 3-fold increase in cGMP can elicit 80% relaxation in the rat aorta (36). Therefore, by allowing more NO to escape from the α globin trap, it is plausible that CYB5R3 blockade could be sufficient for long term beneficial effects. The benefit of reducing CYB5R3 activity could be an ideal tactic to augment NO bioavailability to prevent RBC methemoglobin formation and other important biological reduction reactions that CYB5R3 regulates. More detailed in vivo studies are required to determine ZINC39395747 drug dosing and bio-distribution.

Although these data provide an important first step toward understanding CYB5R3 in vivo, several limitations should be noted. First, one major drawback of the cell and animal studies is the potential off target effects of ZINC39395747 in vivo. Currently, it is unclear whether ZINC39395747 inhibits other NADH reductases, and future work will be required to address this limitation. Furthermore, our studies demonstrate that ZINC39395747 acutely decreases blood pressure and blunts responses to AngII and NE. These effects are acute, and more work will be necessary to understand the effect of long term administration of ZINC39395747 on blood pressure regulation and cardiovascular function. Lastly, to date little is known about CYB5R3 in the cardiovascular system. Therefore, it is imperative that future studies address other cell types and other heme proteins that could be regulated by CYB5R3 as it relates to cardiovascular biology.

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