Mitochondrial Uncouplers: Development as Therapeutics for Metabolic Diseases

Christopher James Garcia

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Webster L. Santos, Chair
Paul R. Carlier
John B. Matson
Andrew N. Lowell

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Abstract

Obesity and its comorbidities have emerged as serious healthcare concerns in the western world due to increased prevalence of nutritional overabundance and decreased physical activity. Due to the significant population affected and economic burden placed on national healthcare systems, there is a demonstrated need for effective weight management therapeutics. Obesity presents clinically diverse phenotypes that increase a person’s susceptibility to comorbidities that commonly result in deteriorated health (cardiovascular disease, diabetes mellitus, hypertension, etc). A comorbidity of specific relevance is non-alcoholic fatty liver disease (NAFLD) and its advanced disease state known as non-alcoholic steatohepatitis (NASH), as it has had a documented rise in prevalence parallel to that observed with obesity. Currently there are no FDA approved therapeutics for NAFLD or NASH, with the majority in clinical development aiming to mitigate the effects caused by accumulation of adipose tissue in the liver known as steatosis. An alternative therapeutic approach is to use small molecules to uncouple oxidative phosphorylation in the mitochondria by passively shuttling protons from the mitochondrial inner membrane space into the mitochondrial matrix. Mitochondrial uncoupling results in the disruption of the proton motive force leading to an upregulation of metabolism (i.e., decrease in steatosis).

Small molecule mitochondrial uncouplers have recently garnered great interest for their potential in treating the advanced disease state of NASH. In this study, we report the structure-activity relationship (SAR) profiling of a 6-amino-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol core, which utilizes the hydroxy moiety as the proton transporter across the mitochondrial inner membrane. We demonstrated that a wide array of substituents are tolerated with this novel scaffold that increased cellular metabolic rates in vitro using changes in oxygen consumption rate as a read-
out. In particular, compound **SHS4121705 (2.12i)** displayed an EC$_{50}$ of 4.3 μM in L6 myoblast cells and excellent oral bioavailability and liver exposure in mice. In the preclinical stelic animal model (STAM) mouse model of NASH, administration of **2.12i** at 25 mg kg$^{-1}$day$^{-1}$ resulted in decreased liver triglyceride levels and improved liver enzymes, NAFLD activity score, and fibrosis without affecting body temperature or food intake. Overall, our initial studies showcased the promise of mitochondrial uncouplers toward the treatment of NASH.

While initial results were promising, the lead compound **2.12i** had reduced potency compared to the alkyl derivatives reported in the SAR, unfortunately alkyl derivatives suffered from poor physiochemical properties, possibly due to metabolism of the alkyl chain. We hypothesized that addressing metabolic liabilities of these compounds could lead to increased potency with maintained efficacy in the STAM mouse model of NASH. Herein, we detail the SAR profiling of a 6-amino-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol core derivatized with 1,1'-biphenyl anilines capable of eliciting mild mitochondrial uncoupling. A wide array of substituents are tolerated, and demonstrated sustained and stable increases in cellular oxygen consumption rates over a broad concentration range. In particular, compound **SHS4091862 (3.9b)** displayed an EC$_{50}$ of 2.0 μM in L6 myoblast cells with a pharmacokinetic profile of C$_{max}$ = 46 μM and t$_{1/2}$ = 4.7 h indicating excellent oral bioavailability. Administration of **3.9b** at 60 mg kg$^{-1}$ day$^{-1}$ in the STAM mouse model of NASH decreased fibrosis, steatosis, and hepatocellular ballooning to result in a 1.9-point decrease in NAFLD activity score (NAS) compared to vehicle. No changes in food intake, body weight, alanine transaminase (ALT) or aspartate transaminase (AST) levels were observed with **3.9b**. Positive control Resmetirom afforded a 1.2-point decrease in NAS score, but increased ALT levels. Cumulatively, our work demonstrates the therapeutic potential of small molecule mitochondrial uncouplers to address metabolic diseases, namely NAFLD.
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General Audience Abstract

There has been a significant increase in the population suffering from metabolic diseases in the western world. Among the most concerning metabolic diseases are obesity and nonalcoholic fatty liver disease, which have been shown to arise from excessive consumption of calorie dense food and limited physical activity. A novel approach to combat these diseases is to use mitochondrial uncouplers that disrupt the body’s natural process for ATP production, causing an increase in metabolism. This increase in the metabolic rate results in the reduction of fat mass including in organs such as the liver. This work describes the design, development, and biological study of mitochondrial uncouplers capable of producing an increase in metabolism; specifically, SHS4121705 (2.12i) and SHS4091862 (3.9b) were shown to be potent uncouplers in vitro and were active in mouse models of fatty liver disease.
Dedication

For my parents, Daniel and Linda Garcia, everything I have done and will do is possible because of the person you raised me to be.
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I thank my advisor Dr. Webster L. Santos for his mentorship and commitment to my development into an independent scientist. He provided me with intellectual guidance while also serving as a prominent role model, demonstrating what it is to be a confident and respected student of life. His ability to make me question every answer I gave while simultaneously communicating his complete confidence in my abilities was both terrifying and essential to nurturing the logical inquisitiveness required of a medicinal chemist.

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Chapter 1: Mitochondrial Uncouplers and Their Therapeutic Applications

1.1 Introduction

Obesity and its comorbidities have emerged as a ubiquitous healthcare concern in the westernized world due to the increased prevalence of nutritional overabundance and decrease in physical activity. As of 2016, 1.9 billion adults, aged 18 and older are overweight, of which 650 million reach the threshold of obese according to the World Health Organization.\(^1\) Due to the significant population affected and the economic burden placed on the national healthcare system, there is a demonstrated need for effective weight management therapeutics. Obesity presents clinically diverse phenotypes commonly resulting in deteriorated health (cardiovascular disease, diabetes mellitus, hypertension, etc), although a minority simply remain at an elevated risk for metabolic syndrome. These serious medical conditions continue to be the focus of medicinal chemistry campaigns as obesity remains the penultimate cause of mortality. However, treating the underlying disease state of obesity needs to be parallel or the comorbidities will continually reemerge. Of specific relevance is non-alcoholic fatty liver disease (NAFLD), which has a documented rise in prevalence parallel to that of obesity. NAFLD displays clinical markers of fatty liver similar to those arising from alcohol abuse and can progress into an aggressive disease state termed non-alcoholic steatohepatitis (NASH). The global incidence of NAFLD is estimated to be 25% among the adult population, a statistic hypothesized to be underreported due to the current cost and invasive nature of diagnosis.\(^2\) Although the understanding of NAFLD is still in its infancy, significant efforts are being put forth to elucidate disease progression and address the unmet public health need. The main goals are to understand the pathophysiology, develop less invasive diagnostic techniques, and provide a pharmaceutical treatment. A potential approach is to use
small molecules to uncouple oxidative phosphorylation in the mitochondria, resulting in increased metabolism.

Diet and exercise regimes have documented effectiveness in combating excessive adipose tissue accumulation and are conceptually simplistic: increase energy expenditure while decreasing caloric intake. Etiologically, adherence to this energy imbalance should result in decreased fat deposits returning to a healthy classification. However, it is not practical in many patient populations in which pharmaceutical/surgical intervention is necessitated. While chronic fat accumulation is systemically detrimental, the liver is significantly affected as it becomes the storage location for excess lipids that cause chronic inflammation. This inflammatory response arises from the recruitment of lymphocytes and macrophages, which infiltrate the liver acini, small cavities in the glands that make up the liver. Cumulatively, excessive calorie intake and inflammation cause a downstream cascade resulting in reciprocal hypertrophic growth of visceral adipose tissue. The long-term effects of chronic liver inflammation is fibrosis and eventually cirrhosis, which requires liver transplantation.

1.2 NAFLD and NASH

1.2.1 Liver Expression

Excessive fat in the liver, known as hepatic steatosis, without signs of inflammation or fibrosis are the defining characteristics of NAFLD. Currently thought to be the liver manifestation of metabolic syndrome, the presence of metabolic syndrome is not a requirement for diagnosis. However, dorsocervical lipohypertrophy is common among those with metabolic syndrome, and is the greatest indicator of a patients risk of NAFLD progressing to NASH. NASH is an advanced form of NAFLD in which irreversible liver damage begins (Figure 1.1). It was first classified in a
Figure 1.1. NALFD progression. As steatosis develops and progresses irreversible liver damage begins to occur. Reprinted with permission from *J. Med. Chem.* 2020, 63, 10, 5031–5073. Copyright 2020 American Chemical Society.

study of 20 patients by the Mayo Clinic in 1980s, where liver biopsies displayed changes congruent with alcohol induced steatohepatitis; alcohol over consumption was markedly absent.

1.2.2 Classification of Nonalcoholic Fatty Liver Disease

Excessive alcohol consumption also contributes substantially to the global burden of the disease, bearing responsibility for 3.8% of deaths worldwide. Excessive alcohol consumption, described as a regular daily intake of 2 and 3 drinks for women and men, respectively, results in deleterious effects on liver structure and function. As the primary site of ethanol metabolism, the parenchymal cells in the liver account for 70% of the organs mass and perform more metabolic functions than any tissue in the body. Alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) are the major enzymes responsible for ethanol metabolism, reaching their half-maximal velocity at circulating concentrations of ethanol well below that required for intoxication.
Nicotinamide adenine dinucleotide (NAD⁺) is the cofactor for ADH-catalyzed ethanol oxidation, which produces NADH and acetaldehyde. Acetaldehyde has been shown to disrupt the activity of macromolecules such as proteins, lipids, and nucleic acids through the formation of covalent bonds.²⁻⁴ At normal levels, the liver mitigates acetaldehyde toxicity through the rapid metabolism of acetaldehyde in the mitochondria via aldehyde dehydrogenase 2 (ALDH2). This redox conversion results in the production of acetate, an essential building block of acetyl-CoA as well as another molecule of NADH. The increased production of NADH disrupts the intracellular ratio of NADH to NAH⁺ resulting in a shift from oxidative metabolism to reductive synthesis.¹⁰ Reductive synthesis generates fatty acids that result in the development of steatosis.

CYP2E1 has an inherently lower catalytic efficiency compared to ADH. however, alcohol consumption increases its hepatocellular expression as ethanol binding promotes a structural conformation and inhibits degradation.⁸ This increased expression of CYP2E1 with chronic alcohol consumption coupled with a 10-fold increase in binding capacity for ethanol, compared to ADH, leads to equally hepatotoxic effects. These effects are attributed primarily to the increased levels of alcohol required for intoxication generating higher concentrations of acetaldehyde, as well as increased production of reactive oxygen species (ROS).¹⁵ ROS production beyond basal levels overwhelms the liver’s capacity to mitigate the destructive capabilities, such as reactions with proteins and unsaturated lipids generating an immune response. Following this cascade, ALD’s clinical representation begins with the formation of steatosis leading to hepatitis and eventually cirrhosis.

Although similar in pathology to ALD, the pathogenesis of NAFLD is disparate and requires a unique therapeutic approach. The historical recognition of NAFLD as the manifestation of metabolic syndrome arises from the shared pathophysiology wherein insulin resistance plays a
pivotal role. Interestingly, the relationship between NAFLD and type II diabetes (T2DM) is more complex, being bidirectional with T2DM implicated as a promoter of NASH development.\textsuperscript{16} Steatosis stands as the primary hepatic insult and diagnostic marker that can maintain a benign course with no correlation to liver related mortality. Although, fatty deposits in the liver can begin to impair function by inducing a cascade of mechanistic changes leading to insulin resistance.\textsuperscript{16} The exact determinates of why some patients experience a progression beyond simple steatosis remains unclear; however, circulating hormones have been implicated with respect to impaired insulin sensitivity.\textsuperscript{17} Adiponectin, a protein hormone adipokine involved in the regulation of glucose levels and fatty acid catabolism, is found at statistically lower levels in NALFD patients compared to those with NASH.\textsuperscript{18} NASH is classified in cases presenting characteristic fatty accumulation with lobular hepatitis in the absence of alcohol consumption.\textsuperscript{19} Lobular hepatitis is spotty hepatocellular necrosis with inflammation, an identical clinical presentation to that of viral hepatitis.\textsuperscript{20} It is now recognized that NASH is an advanced form of NAFLD, an umbrella term used to describe a spectrum of diseases, and has become the most common metabolic liver disease in the world defined by steatosis.\textsuperscript{21} Steatosis is organized into two types, macrovesicular and microvesicular steatosis, the latter being a less common attribute of NAFLD. Macrovesicular steatosis is characterized by a single large fat droplet or multiple smaller droplets which coalesce displacing the nucleus of the hepatocyte to the periphery. Microvesicular steatosis is commonly associated with Reyes syndrome or abuse of drugs and toxins; however, Tandra \textit{et al.} stratified the disorder into three grades: mild, moderate and marked.\textsuperscript{22} These grades are not strictly delineated nor are they restricted to a certain state of NAFLD progression.
1.2.3 *Pathogenesis/Inflammatory Response*

The pathogenesis of NAFLD and NASH is complex and multifactorial, encompassing diverse correlates and cellular responses. Dietary factors like consumption of foods rich in fructose have been implicated in mechanisms of NAFLD progression. As fructose reaches the liver, lipogenesis is initiated, independent of insulin signaling. Fructose-induced lipogenesis causes the increased expression of malonyl-CoA. As malonyl-CoA concentration increases, fatty acids are inhibited from entering the mitochondria, resulting in a decrease of fatty acids available to undergo β-oxidation and subsequent production of acetyl-CoA. Lower concentrations of acetyl-CoA decrease the production of reduced cofactors used to initiate the electron transport chain, slowing mitochondrial ATP production, and increasing reactive oxygen species (ROS) production. As a result, free fatty acids and free cholesterol accumulate in hepatocytes leading to steatosis, which initiates the hepatic inflammatory response, shown in (Figure 1.2).

When efflux of free cholesterol is slowed, the development of foamy Kuffer cells, indicative of NASH, are produced as well as increased cellular sensitivity of transforming growth factor beta (TGF-β), TNF-α, and Fas, which also lead to liver damage. Kuffer cells are the macrophage cells of the liver and have two phenotypes: pro-inflammatory M1 and anti-inflammatory M2. Buildup of free fatty acids in hepatocytes cause cellular stress and initiate the M1 pathway of hepatocyte apoptosis. This stress produces the inflammatory response which further exacerbates apoptosis, the main mechanism of cellular death in current NASH models. TNF-related apoptosis-inducing ligand (TRAIL), Fas and TNFR are all key cell death receptors that are upregulated in NASH, and TNFR1 knockout mice display impaired hepatic insulin action.
Figure 1.2. Hepatic inflammatory response. Steatosis inflicts hepatocyte injury beginning the signaling cascade which produces fibrosis and increased inflammation. Reduction of steatosis will decrease the inflammatory response, reducing further liver injury.\(^\text{25}\)

Hepatocyte apoptosis activates and recruits immune cells, such as Kuffer and hepatic stellate cells. As Kuffer cells engulf apoptotic hepatocytes their phenotype is switched to M1, releasing pro-inflammatory cytokines and chemokines such as INF-γ, TNF-α, TRAIL and Fas that take action to convert hepatocytes into apoptotic hepatocytes. Circulating macrophages, T-cells, and neutrophils are attracted, further promoting hepatocyte cell death and leading to progression of NAFLD and NASH.

The activation of hepatic stellate cells via TGF-β results in secretion of extracellular matrix proteins such as collagen type 1, which form scar tissue, (i.e., fibrosis). These cells reside in the space of Disse, the area between the hepatocyte and the sinusoid, in a quiescent mode. The fibrosis caused by their activation, known as hepatic fibrosis, is a reversible wound healing response that can decrease with loss of injury. However, the chronic nature of inflammation and continual
fibrogenesis associated with NASH leads to the replacement of normal parenchyma with scar tissue, compromising natural liver function and is thus classified as cirrhosis, an irreversible process. Cirrhosis is characterized by two phases: compensated, where parts of the liver are undamaged, and functional or decompensated in which scar tissue has fully covered the organ.

Peroxisome proliferator-activated receptors (PPARs) are also important in the regulation of fatty acid metabolism and inhibition of inflammation.\textsuperscript{29} PPARs, of which there are four ($\alpha, \beta, \delta, \gamma$), heterodimerize with retinoid X receptor (FXR) and then bind to the peroxisome proliferator hormone response elements on target genes. This results in the production of genes involved in fatty acid oxidation. PPAR-$\alpha$ in the liver regulates $\beta$-oxidation, as well as removal of cholesterol and has been shown to be decreased in NAFLD, resulting in steatosis.\textsuperscript{29} PPAR-$\gamma$ in the liver promotes lipogenesis, although it improves insulin sensitivity leading to decreased fibrosis.

As $\beta$-oxidation of lipids slows, lipotoxicity results in an increase of water and calcium influx into the mitochondria, reducing phosphorylation of the voltage-dependent anion-selective channel (VDAC).\textsuperscript{30} The VDAC assumes an open state during conditions of decreased phosphorylation allowing for the release of cytochrome c. Cytochrome c has an important role in the electron transport chain (ETC), but when released into the cytosol it activates caspases, proteolytic enzymes used in cell death. Cytochrome c release also inhibits mitochondrial respiration, resulting in an increased production of ROS. Oxidative stress is now considered to be an important factor in the development of NASH. This is congruent with the findings of Garten \textit{et al.}, showing that when mice are fed a high-fat/high-sucrose diet there is a decrease in hepatic NAD\textsuperscript{+}.\textsuperscript{31}
1.2.4 Disease Progression

While current statistics show NAFLD is commonly found with diseases prevalent in the general population (obesity, diabetes, and metabolic syndrome), little is known about its natural history. Currently, causation is thought to be over-nutrition, poor dietary choices, and an increasingly sedentary lifestyle. Determining why some cases lead to progressive fibrosis while others remain benign remains a gap in knowledge.\textsuperscript{32} If NAFLD continues unchecked, it follows the general progression shown in (Figure 1.3). Chronic inflammation results in fibrosis, rated on a scale of F0 (no fibrosis) to F4 (cirrhosis).\textsuperscript{33} Studies have shown that patients with decompensated cirrhosis displaying progressive fibrosis coupled with Class III obesity, also characterized by
chronic inflammation, are predisposed to developing acute-on-chronic-liver failure (ACLF).\textsuperscript{34} Patients with fibrosis are also at an increased risk of hepatocellular carcinoma.\textsuperscript{35} Progression to cirrhosis is the irreversible replacement of normal liver tissue with scar tissue, impacting normal portal vein blood flow. This results in increased blood pressure and disruption of normal liver function. The associated metabolic symptoms of NAFLD and NASH have a compounding negative effect. Insulin sensitivity decreases, which in turn increases the risk of progression to NASH.\textsuperscript{36} Malet and coworkers have shown that when a patient is diagnosed with NASH, regimes for weight loss result in statistically significant biochemical and histological improvements.\textsuperscript{37}

\textit{1.2.5 Diagnosis and Imaging}

Current diagnostic techniques for NAFLD and NASH are another shortfall in identifying and treating the disease. Liver biopsy is currently the only method for the identification and positive diagnosis of NASH.\textsuperscript{38} Unfortunately, NASH presence, indicated with liver biopsy or current imaging techniques, does not show a direct correlation to the severity of steatosis.\textsuperscript{39} This highlights the need for improved diagnostic and imaging techniques. Less invasive methods are in development, but only provide limited information compared to biopsy. Ultrasound was shown effective in the case of severe hepatic steatosis but displays poor sensitivity when hepatic steatosis is mild.\textsuperscript{40} Measuring liver enzymes and comparing to normal levels is another approach, although it has proven inconclusive thus far due to variability, with many confirmed NAFLD cases presenting levels considered normal.\textsuperscript{41} Angulo and coworkers developed a noninvasive scoring system for NAFLD able to determine the presence of advanced fibrosis.\textsuperscript{42} Holistic approaches are also being pursued using statistical data on obesity and diabetes to build models that apply the suspected incidence of NAFLD. While not applicable to an individual patient’s diagnosis, it helps define the impact of NAFLD on public health as well as highlight areas of need. Recently, another
study was performed using the fatty liver index as a metric for determining NAFLD. This is attractive since relevant data is acquired through routine clinical measurements such as waist circumference, BMI, triglyceride and gamma-glutamyl transferase levels, not requiring invasive techniques. While it benefits from being non-invasive with easy clinical application, it cannot predict changes in fatty liver or diagnose NASH. Another approach is in vivo monitoring of mitochondrial redox metabolism. Along with the deficiency in current diagnostic techniques there are limited therapeutic options for those with NAFLD/NASH. Weight management has proven an effective strategy, although, this option requires significant patient participation and often pharmaceutical intervention is needed.

1.2.6 Potential Pharmaceuticals

Currently, there are no FDA approved drugs to treat NAFLD and NASH. To treat and prevent NASH progression two separate obstacles must be navigated. Least formidable is the associated metabolic complications for which medications exist, second is fibrosis stages 2 - 4. The latter accounts for the gap in medical interventions capabilities. These discrepancies have allowed for a few promising drugs to be given fast-track status by the FDA. Among these Ocaliva, Elafibranor and Selonsertib were estimated to be the first to reach market (Figure 1.4).

Ocaliva (1.1), also commonly known as obeticholic acid (OCA), is the lead compound being pursued by Intercept laboratories and obtained Breakthrough Therapy Status by the FDA. OCA is approved to treat primary biliary cholangitis, and current studies are determining its effectiveness against NASH. It is an agonist for FXR, highly expressed in the liver and, responsible for the suppression of the cholesterol 7 alpha-hydroxylase enzyme which converts cholesterol into bile acid.
Activated FXR promotes small heterodimer partner expression inhibiting transcription of the CYP7A1 gene. Simply, FXR is responsible for maintaining the balance of bile acids in the intestines according to the body’s needs. When bile acid is synthesized, it is transported to the intestine for the purpose of digesting fats by emulsification. Once solubilized, the fat can be transported via the blood and metabolized. Therefore, as OCA activates FXR, there is a decrease in free fatty acids in the blood. FXR interacts with peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is the master regulator of mitochondrial biogenesis and has been shown to interact with PPAR-γ. PPAR-γ controls the process of upregulating/downregulating mitochondrial expression and is responsible for the regulation of fatty acid storage and glucose metabolism. FXR activation represses hepatic lipogenesis and induces fatty acid oxidation, reducing hepatic lipid storage. 49 1.1 failed to obtain FDA approval.
following phase 3 results showing that a common side effect of pruritus was present in a significant patient population.

Elafibranor, (1.2), the lead compound for Genfit, is a dual activator of PPARα/γ, also known as a glitazar. PPAR-α is involved in the regulation of lipid metabolism in the liver and is critical in the process of ketogenesis. PPAR-α is highly expressed in tissues known to oxidize fatty acids, such as the liver and intestine. Its activation lowers serum triglyceride levels and increases high density lipoprotein. Activation of PPAR-γ stimulates lipid uptake, increased expression of ABCA1, and adipogenesis by fat cells. This process forms adipocytes, cells specialized to store energy as fat. ABCA1 is known as the cholesterol efflux protein and is involved in the transport of hepatic cholesterol, resulting in excretion from the liver. While a promising approach for the treatment of NASH, Genfit is no longer pursuing 1.2 as it failed to meet phase 3 primary endpoints of resolving NASH without worsening fibrosis compared to placebo.

Selonsertib, (1.3), the lead compound for Gilead, is an inhibitor of apoptosis signal-regulating kinase 1 (ASK1). ASK1 inhibition prevents downstream phosphorylation by c-Jun N-terminal Kinases (JNKs) and p38 mitogen-activated protein kinases (p38 MAPK), which take part in cellular responses that exacerbate NASH. JNKs and MAPKs are involved in inflammation, cytokine production, cell growth, differentiation, and apoptosis; therefore, inhibition of ASK1 will result in a decreased inflammatory response as well as deregulation of potential fibrotic genes and cell proliferation. Although 1.3 produced dose dependent effects in the phase 3 studies, it failed to reach the primary efficacy endpoint of improving fibrosis without worsening of NASH.

Telmisartan (1.4), an angiotensin II receptor blocker FDA approved for the treatment of hypertension, heart failure and diabetic kidney disease. The mechanism of action interferes with the renin-angiotensin system (RAS), a hormone system responsible for the regulation of blood
pressure and systemic vascular resistance. A hypotensive event causes the levels of plasma renin to increase, catalyzing the conversion of angiotensinogen to angiotensin I. Angiotensin I (AT1) is subsequently converted to angiotensin II (AT2), resulting in potent vasoconstriction and increase in blood pressure. 1.4 has a mixed receptor kinetic profile between surmountable and insurmountable binding to AT2, selectively inhibiting the negative regulation feedback of AT2 on renin secretion.52, 53 Furthermore, AT2 enhances the activation and proliferation of hepatic stellate cells (HSCs) shown to be involved in the progression of hepatic fibrosis.54, 55 Additionally, 1.4 has tangential activity as a partial agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ) a regulator of insulin and glucose metabolism.56 PPAR-γ, when activated, increases the expression of genes responsible for controlling the storage of fatty acids in adipocytes. A decrease in available free fatty acids in circulation results in a metabolic shift in which cells dependence on oxidation of carbohydrates (i.e., glucose) increases; thereby, increasing insulin sensitivity and decreasing hepatic fat accumulation.56

Resmetirom (1.5), is a liver directed and markedly selective thyroid receptor-β (THR-β) agonist being developed by Madrigal pharmaceuticals.57, 58 Selective agonism of THR-β isoform, over THR-α, alleviates concerns of ubiquitous activity responsible for adverse cardiac and bone effects of previous agonist. THR-β is highly expressed in hepatocytes, involved in the regulation of hepatic triglyceride and cholesterol storage and metabolism.59, 60 Thyroid hormone (TH) is involved in the regulation of non-esterified fatty acid uptake, de novo lipogenesis and triglyceride synthesis in hepatic tissue.61 TH has also been shown to stimulate the transcription and activity of adipose triglyceride lipase and hepatic lipase, thereby increasing triglyceride hydrolysis. Free fatty acids are mobilized from triglycerides and subsequently undergo β-oxidation leading to a reduction of hepatic steatosis.59
1.3 Mitochondrial Uncouplers

1.3.1 Oxidative Phosphorylation

A novel approach to treating NAFLD and NASH is the use of mitochondrial uncouplers, which have been previously explored for the treatment of obesity and related metabolic disorders. To appreciate the potential of mitochondrial uncouplers, it is necessary to understand the process of oxidative phosphorylation, shown in (Figure 1.5).

![Figure 1.5. Overview of oxidative phosphorylation and the electron transport chain. Reprinted with permission from J. Med. Chem. 2018, 61, 4641-4655. Copyright 2018 American Chemical Society.](image-url)

In general, oxidative phosphorylation is a process that takes place in the mitochondria and is where cells oxidize nutrients to produce ATP. Pyruvate enters the Krebs cycle and undergoes a series of redox reactions culminating in the production of oxaloacetate to complete the cycle. Four of the eight steps are oxidations producing reduced cofactors NADH and FADH$_2$. Key to this cycle is the starting material, acetyl-CoA, which is produced by either glycolysis or β-oxidation. β-oxidation occurs in the mitochondria and is the catabolic process by which fatty acids are broken...
down. This process is efficient in terms of energy; fatty acids are a highly effective form of energy storage. The complete oxidation of one 16 carbon fatty acid results in the production of 129 ATP molecules.

The cofactors NADH and FADH$_2$ produced from the citric acid cycle can then transfer a pair of electrons to the electron transport chain (ETC). The ETC then functions to ultimately reduce molecular oxygen to water, producing a proton gradient that is used to generate ATP. The donation of electrons is an exergonic process coupled to the endergonic production of ATP. NADH, the higher energy molecule of the two, delivers its electrons to complex I at which point it is oxidized back into NAD$^+$. Complex I catalyzes the reduction of ubiquinone to ubiquinol, releasing energy utilized to pump a proton from the mitochondrial matrix to the intermembrane space. FADH$_2$ is delivered to complex II where it is oxidized back into FAD, the electrons move along the ETC in the same manner although, due to the lower energy, complex II does not pump any protons. The electrons from both complexes are transferred to a mobile electron carrier, ubiquinone (Q), reducing it to QH$_2$. Reduced ubiquinone moves to complex III, releasing energy used to pump protons across the membrane. From complex III the electrons are passed to cytochrome c, a mobile electron carrier, which chauffeurs them to complex IV following the same energy usage as previously described. The ETC concludes when electrons are transferred to molecular oxygen to produce two water molecules, requiring four protons from the matrix and four electrons from the chain.

The proton gradient is not only used to generate ATP; it is also involved in a process known as thermogenesis. Within the mitochondrial inner membrane, there are five uncoupling proteins (UCP1-5), which like ATP synthase are regulated proton channels. However, they are responsible for the generation of heat and are the regulators of mitochondrial respiration. Following the
electrochemical gradient, ions flow from the intermembrane space to the matrix. UCP1 has a role in thermogenesis primarily in the mitochondria of brown adipose tissue. Mice experiments have shown that cold exposure can enhance the transition of white fat to beige adipose tissue.\textsuperscript{63} White fat is used in the storage of extra calories while brown fat dissipates energy through mitochondrial uncoupling, producing heat. Therefore, the ability to convert white fat into beige fat, which has the same potential of brown fat, is an attractive treatment for weight loss. Recent developments in the understanding of how the gut microbiome directly affects the conversion of beige adipose tissue to brown adipose tissue (BAT) provide an avenue as potential alternative strategy.\textsuperscript{64, 65} Non-shivering thermogenesis is the process of heat production to regulate body temperature, through protons bypassing ATP synthase, that results in energy being released as heat illustrating the mechanism of action of mitochondrial uncouplers.

\textit{1.3.2 Small Molecule Uncouplers}

Small molecule mitochondrial uncouplers, also known as protonophores, mimic the activity of UCP's by shuttling protons across the inner membrane of the mitochondria bypassing ATP synthase “uncoupling” oxidative phosphorylation from ATP synthesis (Figure 1.6). Utilizing the pH difference between the intermembrane space (6.8) and mitochondrial matrix (8.1), uncouplers transport protons across the membrane disrupting the proton motive force. Uncouplers are lipophilic weak acids. Lipophilicity is important due to an uncouplers mechanism of action that requires movement through the inner-membrane independent of transporters. A cLogP value is a measure of hydrophilicity/lipophilicity and is calculated as a logarithm of its partition coefficient between n-octanol and water. An assay was developed to measure the degree of mitochondrial uncoupling
Figure 1.6. Mechanism of action for protonophores. Small molecules, represented as hexagons, cross the inner-membrane into the matrix where the acidic proton is released after which they return to the intermembrane space to restart the cycle.

known as an Oxygen Consumption Rate assay (OCR). Uncouplers result in a net increase in consumption of molecular oxygen due to upregulation of the ETC (Figure 1.7).

The most well studied mitochondrial uncouplers are all highly potent lipophilic weak acids, that do not possess tangential mechanisms of action (Figure 1.8). 2,4-Dinitrophenol (DNP, 1.6), first used in the production of ammunition, was enlisted as a diet aid following the observation of weight loss in factory workers experiencing acute exposure.\textsuperscript{67, 68} It was widely used as a diet aid from 1933 to 1938 until it was removed from the market and characterized as unsafe for human consumption.\textsuperscript{69, 70} 1.6 is potent and dose dependent, having a narrow therapeutic window. The compound’s toxicity arises from its ability to depolarize plasma membranes. 1.6 overdose results in death due to hyperthermia, as excessive heat is produced from its uncoupling activity. Although it has a narrow therapeutic window, 1.6 illustrates the potential of uncoupling oxidative phosphorylation, causing an upregulation in the catabolism of nutrients providing beneficial effects in metabolic disorders.\textsuperscript{71} Research into harnessing the potential of 1.6 by increasing the therapeutic window continues.\textsuperscript{72}
Figure 1.7. The Seahorse assay. Oxygen consumption rate (OCR) is measured before and after the addition of inhibitors to derive several parameters of mitochondrial respiration. Initially, baseline cellular OCR is measured, from which basal respiration can be derived by subtracting non-mitochondrial respiration. Next oligomycin, a ATP synthase inhibitor, is added and the resulting OCR is used to derive ATP-linked respiration and proton leak respiration. Next carbonyl cyanide-p-trifluoromethoxy-phenyl-hydrazone (FCCP), a protonophore, is added to collapse the inner membrane gradient and maximal respiratory capacity is derived by subtracting non-mitochondrial respiration from the FCCP rate. Lastly, antimycin A and rotenone, inhibitors of complex III and I, reveal the non-mitochondrial respiration. Mitochondrial reserve capacity is calculated by subtracting basal respiration from maximal respiratory capacity. Reprinted with permission under a Creative Commons Attribution 4.0 License from Reference 66.

Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 1.7), first described by Heytler et al., showed similar uncoupling activity although it was never used as a drug, again due to non-selective depolarization. 1.7 is often used as a standard for uncoupling activity in bioassays and high throughput assays used to detect effects on mitochondrial membrane potential.

Among the most notable mitochondrial uncouplers is BAM15 (1.8), discovered in a high throughput assay of 1450 compounds screening for potential uncouplers of oxidative phosphorylation. 1.8 separates itself from previously reported uncouplers for its ability to utilize
all the mitochondrial respiratory reserve capacity with complete selectivity for the mitochondrial inner membrane, alleviating the toxicity concerns of its predecessors. A subsequent structure activity relationship study revealed that all three ring structures which constitute 1.8 (furazan, pyrazine, aniline) were required for uncoupling activity, implicating the aniline as the source of the acidic proton. Investigation of substituent effects with symmetrical aniline rings showed that 1.8 remained the most promising.\(^6\) Initial \textit{in vivo} studies showed 1.8 was effective in protecting mice from acute renal ischemic-reperfusion injury.\(^5\) Following these promising results, 1.8 was investigated as an anti-obesity therapeutic in a diet induced obesity reversal study in mice.\(^7\) 1.8 was shown to successfully increase nutrient oxidation reducing fat mass with no alteration in food intake or lean tissue loss. Hyperinsulinemic-euglycemic clamp studies showed that 1.8 improved insulin sensitivity further demonstrating the therapeutic promise as an anti-obesity treatment. Further derivatization showed that the unsymmetrical compound 10b (1.9) had increased potency (EC\(_{50} = 190\) nM), demonstrating the first \textit{in vivo} efficacy in a Stelic animal model (STAM) mouse model of NASH.\(^8\) While an increase in potency with maintained efficacy is promising, 1.8 and 1.9 both suffer from poor physiochemical properties, namely solubility and oral bioavailability.

Due to the complex nature of metabolic diseases, a diverse array of pharmaceutical approaches are currently being pursued, most of which have well documented side effects. A novel
approach for the treatment of NASH is to use mitochondrial uncouplers to ameliorate liver histology with limited propensity for side effects due to the mechanism of action relying on the body’s natural metabolic efficiency. Currently the most potent and efficacious mitochondrial uncouplers (1.8 and 1.9) have demonstrated an excellent safety profile with significant improvements in both obesity and NASH.

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Chapter 2: 6-Amino-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol Derivatives as Efficacious Mitochondrial Uncouplers in STAM Mouse Model of Non-alcoholic Steatohepatitis

2.1 Contributions

Novel series of mitochondrial uncouplers was discovered by Joseph M. Salamoun and the structure-activity relationship study was performed in collaboration with Christopher J. Garcia. Dr. Webster L. Santos served as an advisor throughout this study and contributed to the preparation and editing of the final manuscript. The final manuscript was prepared in collaboration between Christopher J. Garcia and Joseph M. Salamoun. This work has been published in Journal of Medicinal Chemistry. Reprinted (adapted) with permission from (Christopher J. Garcia, Joseph M. Salamoun, Stefan R. Hargett, Jacob H. Murray, Sing-Young Chen, Martina Beretta, Stephanie J. Alexopoulos, Divya P. Shah, Ellen M. Olzomer, Simon P. Tucker, Kyle L. Hoehn, and Webster L. Santos J. Med. Chem. 2020, 63, 11, 6203–6224. Copyright (2020) American Chemical Society.

2.2 Abstract

Small molecule mitochondrial uncouplers have recently garnered great interest for their potential in treating nonalcoholic steatohepatitis (NASH). In this study, we report the structure-activity relationship profiling of a 6-amino-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol core, which utilizes the hydroxy moiety as the proton transporter across the mitochondrial inner membrane. We demonstrate that a wide array of substituents is tolerated with this novel scaffold that increased cellular metabolic rates \textit{in vitro} using changes in oxygen consumption rate as a read-out. In particular, compound SHS4121705 (2.12i) displayed an EC$_{50}$ of 4.3 \textmu M in L6 myoblast cells and excellent oral bioavailability and liver exposure in mice. In the STAM mouse model of NASH, administration of 2.12i at 25 mg/kg/day resulted in decreased liver triglyceride levels and
improved liver enzymes (ALT), NAFLD activity score, and fibrosis without affecting body
temperature or food intake. Overall, our studies showcase the promise of mitochondrial uncouplers
toward the treatment of NASH.

2.3 Introduction

Cellular respiration occurs in mitochondria wherein nutrient oxidation leads to the
production of adenosine triphosphate (ATP), an energy rich molecule that fuels cellular function
and signaling pathways.\textsuperscript{1,2} This process, also known as oxidative phosphorylation, occurs by the
generation of a proton motive force (pmf) driven by the efflux of protons from the matrix to the
mitochondrial intermembrane space. The resulting electrochemical gradient drives ATP
production as protons re-enter the mitochondrial matrix via the enzyme ATP synthase. Alternatively, protons may re-enter the mitochondrial matrix independently of ATP synthase, thus bypassing the production of ATP, through a process known as mitochondrial uncoupling.\textsuperscript{3,4} This
proton leak is a natural process in mammals that occurs as both basal and protein-induced leaks.\textsuperscript{5,6}
As proton leak occurs, cells compensate by increasing the metabolic rate to regenerate the proton
motive force and sustain an adequate supply of ATP. Inducible proton leak is a process mediated
by proteins and activated by various stimuli such as oxidative stress or cold exposure that increase
metabolic rate in part through activation of uncoupling proteins (UCPs).\textsuperscript{7-11}

In addition to naturally occurring mitochondrial uncoupling, proton leaks can also be
induced with small molecules that transport protons, by a protonation/deprotonation mechanism,
from the mitochondrial intermembrane space into the mitochondrial matrix.\textsuperscript{4,12-14} Lipophilic weak
acids comprise a major category of protonophores (Figure 2.1). Their ability to modulate cellular
respiration has been explored for therapeutic use, especially for obesity and adult onset diabetes
(T2D).\textsuperscript{7,15-22} Additionally, mitochondrial uncouplers may be useful in the treatment of non-
alcoholic fatty liver disease by slowing down or even preventing hepatic steatosis and nonalcoholic steatohepatitis (NASH) phenotypes.\textsuperscript{19-21, 23, 24, 26} These encouraging results are particularly relevant to today’s clinical challenges as non-alcoholic fatty liver disease (NAFLD) is becoming an epidemic affecting more than 100 million adults and children in the United States alone.\textsuperscript{27-29} The lack of non-invasive diagnostic tools for NASH makes it difficult to confirm the number of people affected; however, the incidence of NASH is increasing and will soon become a significant contributor to the number of liver transplantation.\textsuperscript{30} NASH, an advanced form of NAFLD, is associated with hepatocyte injury with ballooning, lobular inflammation, and often fibrosis and cirrhosis. Due to the lack of FDA-approved drugs for the treatment of NASH, drug discovery in this field is highly active and was the subject of a recent review.\textsuperscript{31}

In addition to increasing energy expenditure, protonophores reduce the production of reactive oxygen species\textsuperscript{32-34} and have been explored for their potential use as antineurodegenerative,\textsuperscript{35} anti-cancer,\textsuperscript{36-39} anti-ageing,\textsuperscript{40-42} and antibacterial agents.\textsuperscript{43} Despite the great preclinical promise of mitochondrial uncouplers for treatment of numerous serious medical conditions,\textsuperscript{44} clinical translation has been challenging due to the promiscuous mechanisms of action of many uncouplers. In fact, any molecule that meets a certain lipophilicity and acidity threshold is potentially an uncoupler, albeit unselectively through both mitochondrial and non-mitochondrial membranes.\textsuperscript{45} Therefore, there is a need for the rational design of novel uncouplers with drug-like properties suitable for translation into the clinic.
Figure 2.1. Structures of select protonophore mitochondrial uncouplers to showcase chemical diversity of the weak acids.

Perhaps the most pertinent example of a mitochondrial uncoupler used in humans, 2,4-dinitrophenol (DNP, 2.1) promoted upwards of 50% increase in metabolism and had weight-loss-inducing effects.46-49 Although widely used as a diet aid in the early 1930’s, DNP was quickly banned by the FDA due to adverse effects including increased body temperature,47, 50-52 cataracts,53-56 blindness,55 and death in some patients.48 Mechanistic studies now indicate that, in addition to the mitochondria, DNP also depolarizes the plasma membrane resulting in a narrow therapeutic window.57-60 FCCP (2), which is highly toxic in animals, has a limited use as an in vitro control in biochemical assays to measure mitochondrial respiration capacity.61-65 In an effort to develop effective strategies to address safety concerns, uncoupling activity was identified in
FDA-approved drugs such as niclosamide (2.3), a well-known anthelmintic drug widely investigated in a number of disease models, most notably cancer for its ability to act on other targets including STAT3, Wnt, and PKA signaling.\textsuperscript{66, 67} Likewise, nitazoxanide (2.4), an antiparasitic drug that acts on pyruvate:ferredoxin oxidoreductase, was recently shown to exhibit uncoupling activity\textsuperscript{68} and is being pursued in a phase 2 clinical trial for NASH-associated fibrosis (ClinicalTrials.gov Identifier: NCT03656068).

Given the broad definition of a protonophore and the lack of a defined biological target, the chemical space is quite vast. As shown in Figure 2.1, phenols (e.g. DNP), dicyano hydrazones (e.g. FCCP), aryl amides (e.g. niclosamide and nitazoxanide),\textsuperscript{68} triazoles (e.g. OPC-163493),\textsuperscript{22} perfluoro alkyl sulfonamides,\textsuperscript{69} benzimidazoles,\textsuperscript{70, 71} and carboranes\textsuperscript{72, 73} are among the diverse class of protonophores studied. Recently, through a high throughput screen, we reported a novel chemical scaffold in BAM15 (2.8), which elicits selective depolarization of the mitochondrial inner membrane.\textsuperscript{74} The mitochondrial-specificity of 2.8 is a unique departure from other protonophores and offers a potential breakthrough to overcome the toxicity of protonophores. Detailed structure-activity relationship (SAR) studies of 2.8 revealed that the oxadiazolo-pyrazine core equipped with aniline moieties are all required for mitochondrial uncoupling activity.\textsuperscript{75} Replacement of the aniline moiety with phenol or alkylation of the aniline N-H resulted in a total loss of activity. This result was an indication that the aniline N-H in 2.8 was the source of the acidic proton.\textsuperscript{76} The [1,2,5]oxadiazolo[3,4-b]pyrazine core is strongly electron withdrawing and is likely the main driver of 2.8’s acidity. Replacing the bicyclic core with pyrazine or triazine, while maintaining two aniline moieties, generally led to lower potency and efficacy.\textsuperscript{77} Interestingly, a subtle change to 2.8 such as regioisomers of the fluoroaniline provided the unsymmetrical analog 2.9 that was efficacious in a streptozotocin (STZ)-induced mouse model of NASH, with
improvements in hepatocyte liver triglyceride content and reduction of inflammation and fibrosis scores.\textsuperscript{78}

Overall, the SAR with the [1,2,5]oxadiazolo[3,4-\textit{b}]pyrazine core and two amine moieties showed a narrow set of tolerated substituents.\textsuperscript{75, 78} The limitations include the solubility and pharmacokinetic properties of these compounds. To improve the physicochemical properties of new derivatives, we replaced the acidic N-H moiety in 2.9 with a hydroxyl functional group (Figure 2.2).\textsuperscript{79} Since O-H is more acidic than N-H, the hydroxyl group may obviate the need for two anilines and widen the scope of tolerated aryl and alkyl substituents. In this study, we performed an SAR investigation of 6-amino-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol derivatives as mitochondrial uncouplers. Our studies identified mitochondrial uncoupler 2.12i that possessed favorable pharmacokinetic properties and desired liver exposure. In a streptozotocin (STZ)-induced mouse model of NASH, 2.12i decreased liver triglyceride levels and improved liver enzymes (ALT), NAFLD activity score, and fibrosis.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Design of 6-amino-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol scaffold as mitochondrial uncoupler.}
\end{figure}

2.4 Results and Discussion

We hypothesized that the acidic proton of the anilines can be replaced by a hydroxyl group since the pK\textsubscript{a} of the hydroxyl group is expected to decrease because of the electron withdrawing
capacity of the furazano pyrazine ring (Figure 2.2). An important intrinsic property of these protonophores is their ability to cycle between the intermembrane space and mitochondrial matrix with concomitant transport of protons; hence, the molecule must be able to penetrate the inner mitochondrial membrane both as a neutral and charged species. Efficient penetration through the membrane is likely facilitated by ‘hiding the negative charge’ rather than anion transport (e.g. via the adenine nucleotide transporter (ANT)) as activity of the parent molecule BAM15 is not affected by the ANT inhibitor carboxyatractyloside;\(^7\) in this case, the oxygen anion is stabilized by delocalization of electrons in the furazan and pyrazine rings through various resonance forms. Furthermore, maintaining one amino group provides a convenient synthetic handle to modulate the lipophilicity of the molecule, another important parameter for membrane crossing. To test this hypothesis, we synthesized and developed the structure-activity relationship profile of a series of 6-amino-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol derivatives.

The synthesis of the pyrazinol derivatives began with the common intermediate 5,6-dichloro-[1,2,5]oxadiazolo[3,4-b]pyrazine (2.11), synthesized as described before (Scheme 2.1).\(^8\) Then in a one-pot reaction, intermediate 2.11 was directly substituted with a sub-stoichiometric equivalent of the requisite alkyl or aryl amines followed by hydroxylation with aqueous potassium hydroxide and then quenching with dilute hydrochloric acid to afford the desired neutral derivatives 2.12. Similarly, a methyl ether derivative 2.13 was synthesized to test the importance of the hydroxyl proton by the one-pot amination of 2.11 followed by reaction with sodium methoxide in methanol.

To investigate the effect of the strongly electron withdrawing furazan ring, pyrazine derivatives were synthesized (Scheme 2.2). Mono-substitution of 2,3-dichloropyrazine (2.14) with benzyl alcohol afforded the desired intermediate 2.15. The aniline moiety was introduced \emph{via} a
**Scheme 2.1.** Reagents and conditions. (a) oxalic acid, 10% HCl, reflux, 4 h, 72%; (b) PCl$_5$, POCl$_3$, 95 °C, 2 h, 77%; (c) one pot reaction: i) amine, Et$_3$N or K$_2$CO$_3$, THF or acetone, rt or 75 °C; ii) KOH$_{(aq)}$ at rt for 2 h; (d) one pot reaction: 4-trifluoromethoxyaniline, Et$_3$N, THF, rt; then NaOMe in MeOH at rt for 1 h.

Buchwald-Hartwig cross-coupling reaction,

With [1,2,5] oxadiazolo[3,4-\textit{b}]pyrazin-5-ol and pyrazine derivatives in hand, we tested their mitochondrial uncoupling activity as a function of oxygen consumption rate (OCR) in L6 rat myoblast cells using an Agilent Seahorse XF analyzer. Briefly, in this assay, L6 myoblasts were monitored over a 90 minute period after treatment with increasing concentrations of compound. OCR increases as mitochondrial uncouplers promote proton leak. BAM15 was used as a positive control because it induces maximal cellular respiration. To benchmark the activity of new analogs, the activities are reported as integrated areas under the curve relative to BAM15 to normalize the data and take into account inter-assay variabilities across the large number of compounds studied. Strong mitochondrial uncouplers will have OCR close to 100% of BAM15 activity and low EC$_{50}$ values.
Scheme 2.2. Reagents and conditions. (a) NaH, BnOH, THF, rt, 2 h, 87%; (b) i. Pd$_2$dba$_3$ (10 mol%), Xantphos (10 mol%), arylamine (1.3 equiv.), K$_2$CO$_3$ (2.3 equiv.), dioxane, 110 °C, 16 h; ii. 10% Pd/C (5 mol %), H$_2$ (g) (1 atm), MeOH, rt, 1.5 h, 12-37% over two steps.

Inspired by previous work on 2.9,$^{78}$ fluorinated analogs in the *ortho*, *meta* and *para* positions were synthesized. The unsymmetrical 2.9 contains a 2-fluoroaniline and 3-fluoroaniline that stimulates respiration in L6 rat myoblast cells with improved EC$_{50}$ value (190 nM) relative to BAM15. The first analog 2.12a, a direct derivative of 2.9 containing a 2-fluoroaniline, retained 74% of BAM15 uncoupling capability, albeit at a 40x loss of potency (Table 2.1). The corresponding *meta* (2.12b) and *para* (2.12c) derivatives were likewise effective although with decreased activity. Nonetheless, the activities were sufficiently compelling from this chemical scaffold. From the handling of these compounds during synthesis and purification, it was apparent that the physical properties (polarity and solubility) were significantly different from 2.9. Taken together, an extensive SAR profiling of novel derivatives were pursued (Tables 2.1-5).

The first series of analogs incorporated a diverse array of electron withdrawing groups (Table 2.1). A variety of halogenated moieties were well-tolerated including trifluoromethyl (2.12d–2.12f), trifluoromethoxy (2.12g and 2.12i), OCF$_2$CHF$_2$ (2.12m), chloro (2.12j and 2.12k) and difluorobenzodioxole (2.12o), which all had >60% of BAM15 OCR and an EC$_{50}$ ≤ 13 µM. The OCF$_3$ analogs (2.12g–2.12i) were the most promising with activity in the single digit micromolar range and the *para*-substituted derivative 2.12i having the best potency EC$_{50}$ at 4.3 µM. Anilines with multiple halogenated groups (2.12q–2.12ab) were less tolerated except for 2.12v; however, this compound is > 2 fold less potent than 2.12i.
Table 2.1. Oxygen consumption rates of halogenated aniline derivatives in L6 rat myoblast cells.\(^a\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR(^b)</th>
<th>EC(50), µM</th>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR(^b)</th>
<th>EC(50), µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAM15</td>
<td>-</td>
<td>100%</td>
<td>0.31 ± 0.06</td>
<td>2.12p</td>
<td>-</td>
<td>29%</td>
<td>34.0 ± 8.0</td>
</tr>
<tr>
<td>2.9</td>
<td>-</td>
<td>71%</td>
<td>0.19 ± 0.01</td>
<td>2.12q</td>
<td>-</td>
<td>45%</td>
<td>9.7 ± 1.8</td>
</tr>
<tr>
<td>2.12a</td>
<td>-</td>
<td>74%</td>
<td>12.5 ± 0.8</td>
<td>2.12r</td>
<td>-</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.12b</td>
<td>-</td>
<td>67%</td>
<td>31.2 ± 2.0</td>
<td>2.12s</td>
<td>-</td>
<td>46%</td>
<td>23.4 ± 4.2</td>
</tr>
<tr>
<td>2.12c</td>
<td>-</td>
<td>51%</td>
<td>38.6 ± 4.8</td>
<td>2.12t</td>
<td>-</td>
<td>47%</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>2.12d</td>
<td>-</td>
<td>88%</td>
<td>10.6 ± 0.7</td>
<td>2.12u</td>
<td>-</td>
<td>30%</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>2.12e</td>
<td>-</td>
<td>66%</td>
<td>13.3</td>
<td>2.12v</td>
<td>-</td>
<td>76%</td>
<td>11.6 ± 1.2</td>
</tr>
<tr>
<td>2.12f</td>
<td>-</td>
<td>68%</td>
<td>10.4 ± 1.4</td>
<td>2.12w</td>
<td>-</td>
<td>32%</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>2.12g</td>
<td>-</td>
<td>67%</td>
<td>7.1 ± 0.5</td>
<td>2.12x</td>
<td>-</td>
<td>10%</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>2.12h</td>
<td>-</td>
<td>47%</td>
<td>5.1 ± 0.4</td>
<td>2.12y</td>
<td>-</td>
<td>27%</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>2.12i</td>
<td>-</td>
<td>64%</td>
<td>4.3 ± 0.7</td>
<td>2.12z</td>
<td>-</td>
<td>32%</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>2.12j</td>
<td>-</td>
<td>84%</td>
<td>6.3 ± 0.7</td>
<td>2.12aa</td>
<td>-</td>
<td>26%</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>2.12k</td>
<td>-</td>
<td>72%</td>
<td>11.7 ± 1.3</td>
<td>2.12ab</td>
<td>-</td>
<td>8%</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

\(^a\) Data from [Shi et al., 2019](https://doi.org/10.1007/s10895-019-02788-7)
Interestingly, both the aniline N-H and O-H were required for activity. To determine the effect of acidic protons in N-H and O-H in the scaffold of 2.12a, the corresponding methylated versions were synthesized. N-methylated analog 2.12ae was completely devoid of uncoupling activity. Likewise, methylation of the phenolic oxygen (2.13) significantly abrogated the compound’s activity suggesting the role of the hydroxyl group as a protonophore.

After the encouraging result from the OCF₃ analog 2.12i, a series of alkoxy analogs was generated (Table 2.2). Whereas the methoxy analog 2.12af was moderately efficacious, the butoxy analogs (2.12ag–2.12ah) were significantly more potent than the methoxy derivative (2.12af), presumably due to the increased lipophilicity favoring permeation of the mitochondrial membrane. Because phenylalkylether groups are known to be a metabolic liability, fluorinated analogs were introduced (2.12ai–2.12an) to potentially enhance metabolic stability. However, many of these compounds showed diminished efficacy or potency. The cyclopropyl methoxy analog 2.12ao, a four carbon derivative of the butoxy chain in 2.12ah and 2.12an, had a promising result with 60% of BAM15 OCR and an EC₅₀ of 1.8 µM. Additionally, the benzyl derivative 2.12ar had good activity and potency. The pentafluorosulfanyl group (2.12at and 2.12au), which is a proposed
isostere for the trifluoromethyl group, and 2.12av, the sulfur derivative of 2.12i, had low efficacy. The electron withdrawing sulfonyl moiety (2.12as) and a cyano group (2.12aw) were not tolerated.

Table 2.2. Oxygen consumption rates of alkoxy, cyano, and thio-aniline derivatives in L6 rat myoblast cells.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR</th>
<th>EC50, µM</th>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR</th>
<th>EC50, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.12af</td>
<td>OCH3</td>
<td>41%</td>
<td>75.4 ± 9.3</td>
<td>2.12ao</td>
<td></td>
<td>60%</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>2.12ag</td>
<td>H</td>
<td>55%</td>
<td>2.3 ± 0.2</td>
<td>2.12ap</td>
<td></td>
<td>27%</td>
<td>46.9 ± 4.8</td>
</tr>
<tr>
<td>2.12ah</td>
<td>O</td>
<td>56%</td>
<td>3.7 ± 1.4</td>
<td>2.12aq</td>
<td></td>
<td>44%</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>2.12ai</td>
<td>OCH3</td>
<td>50%</td>
<td>42.3 ± 8.3</td>
<td>2.12ar</td>
<td></td>
<td>66%</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>2.12aj</td>
<td>CH3</td>
<td>57%</td>
<td>7.0 ± 0.7</td>
<td>2.12as</td>
<td>3SO2Me</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.12ak</td>
<td>OCF3</td>
<td>60%</td>
<td>10.4 ± 1.2</td>
<td>2.12at</td>
<td></td>
<td>29%</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>2.12al</td>
<td>OCF3</td>
<td>43%</td>
<td>4.0 ± 0.3</td>
<td>2.12au</td>
<td></td>
<td>46%</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>2.12am</td>
<td>OCF3</td>
<td>24%</td>
<td>4.2 ± 0.2</td>
<td>2.12av</td>
<td></td>
<td>24%</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>2.12an</td>
<td>OCF3</td>
<td>46%</td>
<td>2.1 ± 0.5</td>
<td>2.12aw</td>
<td></td>
<td>NA</td>
<td>--</td>
</tr>
</tbody>
</table>

*a* Highest tested concentration is 200 µM.  
*b* Ratio of integrated area under OCR dose curve above baseline relative to that of BAM15. NA = No Activity.

We next investigated the effect of other rings as well as alkyl groups on the aniline ring (Table 2.3). A sharp decrease in potency was observed with aniline derivative 2.12ax and N-methylation (2.12ay). In stark contrast to our prior SAR studies, the alkyl anilines were well-
Table 2.3. Oxygen consumption rates (OCR) of alkyl substituted aniline derivatives in L6 rat myoblast cells.a

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCRb</th>
<th>EC_{50}, µM</th>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCRb</th>
<th>EC_{50}, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.12ax</td>
<td>( \text{NH} )</td>
<td>52%</td>
<td>66.7 ± 22.7</td>
<td>2.12bk</td>
<td>( \text{NH} )</td>
<td>51%</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>2.12ay</td>
<td>( \text{NH} )</td>
<td>NA</td>
<td>--</td>
<td>2.12bl</td>
<td>( \text{NH} )</td>
<td>33%</td>
<td>4.7 ± 3.0</td>
</tr>
<tr>
<td>2.12az</td>
<td>( \text{NH} )</td>
<td>69%</td>
<td>4.0 ± 0.5</td>
<td>2.12bm</td>
<td>( \text{F} )</td>
<td>65%</td>
<td>6.7 ± 1.6</td>
</tr>
<tr>
<td>2.12ba</td>
<td>( \text{NH} )</td>
<td>66%</td>
<td>20.4 ± 3.9</td>
<td>2.12bn</td>
<td>( \text{F} )</td>
<td>14%</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>2.12bb</td>
<td>( \text{NH} )</td>
<td>64%</td>
<td>51.9 ± 10.2</td>
<td>2.12bo</td>
<td>( \text{F} )</td>
<td>5%</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>2.12bc</td>
<td>( \text{NH} )</td>
<td>14%</td>
<td>&gt;100</td>
<td>2.12bp</td>
<td>( \text{F} )</td>
<td>5%</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>2.12bd</td>
<td>( \text{NH} )</td>
<td>26%</td>
<td>3.0 ± 0.2</td>
<td>2.12bq</td>
<td>( \text{OCF}_3 )</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.12be</td>
<td>( \text{NH} )</td>
<td>74%</td>
<td>25.2 ± 9.1</td>
<td>2.12br</td>
<td>( \text{OCF}_3 )</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.12bf</td>
<td>( \text{NH} )</td>
<td>63%</td>
<td>10.7 ± 1.9</td>
<td>2.12bs</td>
<td>( \text{OH} )</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.12bg</td>
<td>( \text{NH} )</td>
<td>73%</td>
<td>2.2 ± 0.3</td>
<td>2.12bt</td>
<td>( \text{CH}_3 \text{C}_6H_4 )</td>
<td>86%</td>
<td>32.8 ± 8.0</td>
</tr>
<tr>
<td>2.12bh</td>
<td>( \text{NH} )</td>
<td>81%</td>
<td>3.9 ± 1.0</td>
<td>2.12bu</td>
<td>( \text{CH}_3\text{C}_6H_4\text{OCF}_3 )</td>
<td>69%</td>
<td>4.6 ± 0.9</td>
</tr>
<tr>
<td>2.12bi</td>
<td>( \text{NH} )</td>
<td>18%</td>
<td>112.8 ± 30.4</td>
<td>2.12bv</td>
<td>( \text{CN} )</td>
<td>23%</td>
<td>35.7 ± 5.5</td>
</tr>
<tr>
<td>2.12bj</td>
<td>( \text{NH} )</td>
<td>76%</td>
<td>2.1 ± 0.3</td>
<td>2.12bw</td>
<td>( \text{O} )</td>
<td>42%</td>
<td>93.7 ± 16.9</td>
</tr>
</tbody>
</table>

\(^a\)Highest tested concentration is 200 µM.

\(^b\)Ratio of integrated area under OCR dose curve above baseline relative to that of BAM15. NA = No Activity.
tolerated in the absence of any halogens or strongly electron withdrawing groups. More specifically, anilines containing isomers of butane were among the most efficacious and potent,

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCRb</th>
<th>EC50, µM</th>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCRb</th>
<th>EC50, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.12bx</td>
<td>H</td>
<td>NA</td>
<td>--</td>
<td>2.12cd</td>
<td>63%</td>
<td>38.3 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>2.12by</td>
<td>H</td>
<td>19%</td>
<td>3.4 ± 0.3</td>
<td>2.12ce</td>
<td>60%</td>
<td>7.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>2.12bz</td>
<td>16%</td>
<td>65.5 ± 10.2</td>
<td></td>
<td>2.12cf</td>
<td>42%</td>
<td>22.8 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>2.12ca</td>
<td>CF3</td>
<td>8%</td>
<td>109.8 ± 45.8</td>
<td>2.12cg</td>
<td>23%</td>
<td>2.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>2.12cb</td>
<td>H</td>
<td>20%</td>
<td>53.2 ± 11.3</td>
<td>2.12ch</td>
<td>NA</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>2.12cc</td>
<td>OCH3</td>
<td>32%</td>
<td>13.4 ± 4.1</td>
<td>2.12ci</td>
<td>43%</td>
<td>4.9 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

*Highest tested concentration is 200 µM.

*Ratio of integrated area under OCR dose curve above baseline relative to that of BAM15. NA = No Activity.

e.g. tert-butyl (2.12bg and 2.12bh) and n-butyl (2.12bj). Unfortunately, the half-life of these analogs in mice is short (< 1 h) as alkyl moieties are susceptible to ω-oxidation (Table 2.6). To circumvent this issue, fluorine groups were added to potentially enhance the metabolic stability. Interestingly, with the exception of 2.12bm and 2.12bu, the combination of halogens and alkyl groups (2.12bn–12br, and 2.12bt) led to poor activity similar to the fluorinated alkoxy analogs. The incorporation of additional polar groups such as a primary alcohol (2.12bs), a cyano group (2.12bv) or a ketone (2.12bw) were not tolerated.
We next investigated whether alkyl amines were effective as mitochondrial uncouplers (Table 2.4). Exchanging the aniline with alkyl and cycloalkyl groups resulted in greatly diminished efficacy and potency with the exception of 1-adamantyl amine (2.12ce) with 60% of BAM15 OCR and an EC₅₀ value of 7.0 µM. The analog 2.12ce was selected for pharmacokinetic studies as a representative of alkyl amines (Table 2.6).

Finally, we determined the effect of the furazan ring (Table 2.5). Replacing the [1,2,5]oxadiazolo[3,4-b]pyrazine core with a pyrazine resulted in complete loss of activity. Unfortunately, all compounds synthesized bearing fluoro (2.16a), alkyl (2.16b), electron withdrawing groups (2.16c–2.16f), and biphenyls (2.16g and 2.16h) were inactive. These results highlight the significance of the electron withdrawing effect of the bicyclic core.

**Table 2.5.** Oxygen consumption rates (OCR) of pyrazine derivatives in L6 rat myoblast cells.ª

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCRb</th>
<th>EC₅₀, µM</th>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCRb</th>
<th>EC₅₀, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.16a</td>
<td>-N-F</td>
<td>NA</td>
<td>--</td>
<td>2.16e</td>
<td>-N-CH₃</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.16b</td>
<td>-N-C₆H₄</td>
<td>NA</td>
<td>--</td>
<td>2.16f</td>
<td>-N-CF₃</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.16c</td>
<td>-N-C₆H₄</td>
<td>NA</td>
<td>--</td>
<td>2.16g</td>
<td>-N-OC₆H₄</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>2.16d</td>
<td>-N-CF₃</td>
<td>NA</td>
<td>--</td>
<td>2.16h</td>
<td>-N-OCF₂</td>
<td>NA</td>
<td>--</td>
</tr>
</tbody>
</table>

ªHighest tested concentration is 200 µM.
b Ratio of integrated area under OCR dose curve above baseline relative to that of BAM15. NA = No Activity.

Based on the OCR assay, we selected representative compounds to determine their suitability for *in vivo* studies in a mouse model of NASH. Thus, mice were dosed with 10 mg/kg
Table 2.6. Pharmacokinetic profile of select analogs in mice.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>( C_{\text{max}} ) (µM)</th>
<th>( t_{1/2} ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.12i (SHS4121705)</td>
<td><img src="image" alt="Structure" /></td>
<td>80.9</td>
<td>5.7</td>
</tr>
<tr>
<td>2.12ah</td>
<td><img src="image" alt="Structure" /></td>
<td>3.4</td>
<td>0.3</td>
</tr>
<tr>
<td>2.12az</td>
<td><img src="image" alt="Structure" /></td>
<td>12.5</td>
<td>0.4</td>
</tr>
<tr>
<td>2.12bh</td>
<td><img src="image" alt="Structure" /></td>
<td>22.3</td>
<td>0.6</td>
</tr>
<tr>
<td>2.12bj</td>
<td><img src="image" alt="Structure" /></td>
<td>12</td>
<td>0.7</td>
</tr>
<tr>
<td>2.12ce</td>
<td><img src="image" alt="Structure" /></td>
<td>18</td>
<td>1.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Compounds were administered at 10 mg/kg body weight by oral gavage to male mice. Plasma samples collected over 8-24 h were analyzed by LC-MS/MS. \( C_{\text{max}} \) = maximal plasma concentration. \( t_{1/2} \) = half-life.

of compound per oral and pharmacokinetic parameters were determined. As shown in Table 2.6, the majority of the compounds had poor oral bioavailability (< 22 µM) and short half-life (1.1 h or less). Fortunately, 2.12i (SHS4121705) resulted in a peak plasma concentration of ~81 µM and a half-life of 5.7 h after administering by oral gavage (Figure 2.3A).

We then evaluated the effect of SHS4121705 on core body temperature as a function of dose at 5, 20, 50 mg/kg or vehicle control by oral gavage. As shown in Figure 2.3B, there is no effect on core body temperature detected by rectal probe thermometer at up to 4 hours after an acute dose. This is an important safety parameter as increased body temperature is a characteristic of some toxic protonophores such as DNP.\textsuperscript{49} In addition, tissue distribution of SHS4121705 in the liver is crucial as complications of NASH are manifested in the liver. After dosing animals with 10 mg/kg of SHS4121705 by oral gavage, mice were sacrificed 1 hour after exposure and levels
Figure 2.3. Acute dose studies of **SHS4121705**. To measure pharmacokinetics (A) **SHS4121705** was administered at 10 mg/kg body weight by oral gavage and plasma samples were collected at the time points shown and analyzed by LC-MS/MS. Body temperature (B) was measured using a rectal temperature probe following an acute dose of 5-50 mg/kg **SHS4121705**, or vehicle. No significant changes were detected in body temperature, as assessed by Two-Way Repeated Measures ANOVA. Tissue distribution (C) of **SHS4121705** was measured at 1 hour after an acute oral gavage of at 10 mg/kg in C57BL/6 male mice. Compound concentration was measured in tissues (liver, gonadal white adipose tissue (WAT), mixed quadriceps muscle (Quad), kidney, heart and brain) by LC-MS/MS. n=3 C57BL/6 male mice for all experiments.

of compound were determined by liquid chromatography tandem mass spectrometry (LC/MS-MS). As illustrated in Figure 2.3C, **SHS4121705** exposure was highest in the liver (15.8 ± 1.3 µg/g) and kidney (9.2 ± 1.2 µg/g). The favorable PK properties of **SHS4121705** is supported by the improvement of physicochemical properties (solubility in PBS, pH 7.4: >500 µM; cLogP = 5.2) when compared to starting lead compound 9 (solubility in PBS, pH 7.4: <10 µM; cLogP = 6.4). We also found that **SHS4121705** has no activity on ion channels such as hERG. Taken together, the favorable physicochemical properties, excellent oral bioavailability and liver exposure suggest that **SHS4121705** was a suitable candidate for *in vivo* efficacy studies against NASH.

The *in vivo* efficacy was determined using the Stelic animal model (STAM) of NASH with Telmisartan as a positive control, as described before.78,90 Over the course of 21 days, **SHS4121705** was administered with food at a dose of 25 mg/kg/d. Telmisartan was administered by oral gavage in water once daily at a dose of 10 mg/kg. Food intake was recorded during the treatment period from mice grouped 4 per cage with 2 cages per treatment group (i.e., 8 mice/treatment group). **SHS4121705** showed no effect on food consumption (331 ± 27 g/cage) as
compared to vehicle (318 ± 13 g/cage), whereas the telmisartan group showed a 9% decrease in food intake (246 ± 2 g/cage). As shown in Figure 2.4A-B, mice treated with SHS4121705 had a lower fibrosis score measured by Picrosirius Red stained area (0.62 ± 0.07%) compared to untreated vehicle control (0.91 ± 0.09%). SHS4121705 anti-fibrosis efficacy was similar to the positive control telmisartan (0.60 ± 0.07%). Mice treated with SHS4121705 had a 2-point improvement in NAS score compared to vehicle control mice (Figure 2.4C, 2.63 ± 0.18 compared to 4.63 ± 0.26 in vehicle), resulting from a lower score in all three liver NAS markers: steatosis (0 ± 0), inflammation (2.38 ± 0.18) and ballooning (0.25 ± 0.16) when compared to vehicle (steatosis 1.0 ± 0), inflammation (2.75 ± 0.16), ballooning (0.88 ± 0.23) (Figure 2.4D-F). Importantly, a complete reduction in steatosis score was observed in SHS4121705 treated mice. Ballooning score was also decreased by SHS4121705, which is notable as early studies have indicated that ballooning is one of the few histological features associated with risk of cirrhosis development in NAFLD. Additionally, ballooning is linked to the attraction of inflammatory cells, suggesting that longer in vivo studies, that allow for greater accumulation of inflammatory cells in the vehicle group, could show a further reduction in inflammation score. The impact of SHS4121705 on whole body physiology was investigated, by measuring several parameters of body composition and biochemistry.

Unlike telmisartan-treated animals, there was no change in body weight or liver weight following SHS4121705 treatment (Figure 2.5A-C). Both SHS4121705 and telmisartan decreased liver triglyceride content as determined from biochemical assay of liver tissue (Figure 2.5D). SHS4121705 did not alter plasma triglyceride, cholesterol or glucose levels, while telmisartan raised plasma cholesterol and glucose (Figure 2.5E-G). Both SHS4121705 and telmisartan improved the primary serum marker of liver damage, alanine aminotransferase (ALT) (Figure
2.5H) without altering levels of aspartate aminotransferase (AST) (Figure 2.5I). Overall, the physiological data show that compound SHS4121705 decreased biochemical markers of liver.

**Figure 2.4.** Histological results from liver tissue of STAM mice. Representative images of hematoxylin & eosin and Sirius Red staining of fixed tissue (A). Fibrosis was measured by quantification of Sirius Red-positive area (B). NAFLD Activity Scores (C) were calculated from pathologist scoring of steatosis (D), inflammation (E) and ballooning (F). * indicates p<0.05. Statistical significance was assessed by Kruskal-Wallis test. n=8 male mice per group.
Figure 2.5. Physiological parameters of STAM mice. Telmisartan, but not SHS4121705, decreased body weight (A), liver weight (B) and liver-to-body weight ratio (C). Both drug treatments decreased liver triglyceride (D). SHS4121705 did not change plasma triglyceride (E) or cholesterol (F) levels, nor whole blood glucose (G). SHS4121705 and Telmisartan treatment decreased plasma ALT (H) but had no effect on plasma AST (I). * indicates p<0.05. Statistical significance assessed by One-Way ANOVA for normally distributed data (D, G, I) and Kruskal-Wallis test for non-parametric data (A-C, E-F, H). n=8 male mice per group.

damage and decreased steatosis, inflammation and ballooning resulting in a 2-point improvement in NAS score. These results suggest that mitochondrial uncouplers have potential as NASH therapeutics.

2.5 Conclusions

Mitochondrial uncouplers have been shown to increase cellular respiration, uncoupling oxidative phosphorylation and nutrient metabolism. Therapeutic application of uncouplers has been supported in a number of preclinical disease models including obesity and fatty liver disease. Promising drug candidates include the FDA-approved drug niclosamide, which is currently being repurposed and has efficacy in fatty liver models, although maintaining a number of off-target
effects. Our discovery of [1,2,5]oxadiazolo[3,4-b]pyrazine derivatives point to a new class of mitochondria-selective uncouplers with \textit{in vivo} efficacy in a mouse model of NASH. In this study, we focused on containing the [1,2,5]oxadiazolo[3,4-b]pyrazine core and replacing one of the aniline moieties with a hydroxyl group to expand the tolerated chemical space and enable modulation of physical properties. Our investigations revealed that this new modification preserved the protonophoric activity with both electron withdrawing and electron donating groups tolerated. Electron donating alkyl groups were among the most potent and efficacious; however, electron withdrawing groups approached the same potency and efficacy with greatly improved pharmacokinetic properties. In particular, we discovered that \textbf{SHS4121705} (2.12i) had excellent oral bioavailability and exposure in liver that encouraged the advancement into \textit{in vivo} efficacy studies. In the STAM mouse model of NASH, \textbf{SHS4121705} improved NAFLD activity score as expected of an anti-NASH agent, decreased liver triglyceride levels, and decreased liver ALT and fibrosis without affecting body temperature or food intake. Taken together, these results support the continued development of this novel scaffold of mitochondrial uncouplers as potential agents for the treatment of NASH.

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Chapter 3: 6-([1,1'-biphenyl]-4-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol Derivatives Elicit Mild Mitochondrial Uncoupling Effective in Combating NASH

3.1 Contributions

This chapter is an adaptation of a manuscript currently being written by the author, Joseph M. Salamoun, Dr. Kyle L. Hoehn of the University of New South Wales, and Dr. Webster L. Santos for the publication in a peer-reviewed Journal. The structure-activity relationship study was performed by Christohper J. Garcia in collaboration with Joseph M. Salamoun. Dr. Webster L. Santos served as an advisor throughout this study and contributed to the preparation and editing of the final manuscript. Jacob H. Murray and Robert J. Grams contributed to the preparation of compounds. All biological studies were performed by Sing-Young Chen, Martina Beretta, Stephanie J. Alexopoulos, Divya P. Shah, Ellen M. Olzomer.

3.2 Abstract

Small molecule mitochondrial uncouplers have recently emerged as a novel approach for treating nonalcoholic steatohepatitis (NASH). Herein, we present the structure-activity relationship profiling of a 6-amino-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol core, derivatized with 1,1'-biphenyl anilines capable of eliciting mild mitochondrial uncoupling. A wide array of substituents are tolerated, demonstrating sustained and stable increases in cellular oxygen consumption rates over a broad concentration range. In particular, compound SHS4091862 (3.9b) was orally bioavailable and displayed an EC_{50} of 2.0 μM in L6 myoblast cells with a pharmacokinetic profile of C_{max} = 46 μM and t_{1/2} = 4.7 h. Administration of 3.9b at 60 mg kg^{-1} day^{-1} in the STAM mouse model of NASH decreased fibrosis, steatosis, and hepatocellular ballooning to result in a 1.9-point decrease in NAFLD activity score (NAS) score compared to vehicle. No changes in food intake, body weight, ALT or AST levels was observed with 3.9b.
Positive control Resmetirom afforded a 1.2-point decrease in NAS score, but increased ALT levels. These results demonstrate the promise in utilizing mild mitochondrial uncoupling as a potential therapeutic approach in treating NASH.

3.3 Introduction

Non-alcoholic steatohepatitis (NASH) is the advanced form of the chronic heterogeneous disease known as non-alcoholic fatty liver disease (NAFLD), an epidemic affecting an estimated 25% of the global population. NAFLD describes a spectrum of liver diseases ranging from simple steatosis (accumulation of fat in liver) to non-alcoholic steatohepatitis (NASH) and eventually cirrhosis, which is forecasted to rapidly become the leading cause of liver transplantation. The environmental and biological factors contributing to hepatic steatosis have been thoroughly investigated, although the mechanisms underlying why some patients progress beyond simple steatosis to NASH complicated with fibrosis remains to be fully understood. Considered a multisystem disease, NAFLD patients are likely to have a comorbid diagnosis of obesity, type 2 diabetes mellitus, metabolic syndrome or chronic kidney disease. There is well-documented commonality in the associated risk factors between these disease states, suggesting that management of one will have beneficial effects on comorbidities. Steatosis is the primary hepatocyte insult underlying an increase of inflammation and ballooning, both of which are associated with an increased risk of fibrosis development. Due to the significant association of fibrosis to liver failure and patient mortality, fibrosis prevention or reduction is the main goal of many drug discovery campaigns. The multifactorial nature of NASH is evidenced by the diverse mechanisms of action for therapeutic agents in clinical development and by the lack of an FDA-approved pharmacotherapy. The current standard of care for advanced NAFLD is lifestyle interventions such as exercise, diet, and weight loss.
Figure 3.1. Structures of potential NASH therapeutics.

However, lifestyle modification regimens suffer greatly from patient non-compliance and are not a viable approach in some patient populations.\textsuperscript{22, 23}

Current therapeutic strategies in clinical development target either fibrosis or NASH, or both. NASH is scored histopathologically on a scale from 0-8 by the degree of steatosis (up to 3 points), hepatocyte ballooning (up to 2 points), and lobular inflammation (up to 3 points). Thus, a pre-treatment biopsy is typically required for definitive NASH diagnosis and a post-treatment biopsy is required to determine the degree of therapeutic efficacy.\textsuperscript{24-26} One of the leading candidates in Phase III clinical trials is Resmetirom (3.1), a thyroid hormone receptor (THR)-$\beta$-selective agonist developed by Madrigal Pharmaceuticals, which increases hepatic lipid metabolism (Figure 3.1).\textsuperscript{27, 28} The Phase 2b primary endpoint, reduction of hepatic fat, was met with only mild adverse events of diarrhea and nausea reported.\textsuperscript{29} Cenicriviroc (3.2) is a C-C
chemokine receptor (CCR) 2 and 5 antagonist in development by Allergan with anti-inflammatory and antifibrotic activity. While initial results of Phase 2b studies showed decreased fibrosis without worsening of steatohepatitis, final data suggest that no further improvement was observed after the first year compared to placebo. Elafibranor (3.3), a dual peroxisome proliferator-activated receptor PPARα and PPARγ agonist in development by Genfit, recently showed no statistical improvement compared to placebo control in its Phase III NASH trial. Thus, alternative strategies to treat NASH are urgently needed.

A complement to targeted therapeutics, where a specific cellular signaling pathway or function is modulated, is the promotion of an otherwise natural systemic process such as mitochondrial uncoupling. Given the complex and heterogeneous pathology of NASH, a systemic approach to target energy metabolism might be more suitable. This is already demonstrated by the effectiveness of diet and exercise induced weight loss in humans, e.g., multiple studies have shown that a ≥10% reduction of body weight resulted in fibrosis regression. Mitochondria are responsible for the regulation of nutrient metabolism with the principal role of providing the high energy molecule adenosine triphosphate (ATP) to fuel cellular functions. Catabolic pathways utilize carbohydrates, lipids, and proteins to generate an electrochemical gradient, known as the proton motive force (PMF), across the mitochondrial inner membrane. The electrochemical gradient provides the driving force whereby protons pass through ATP synthase in the mitochondrial inner membrane to reach the mitochondrial matrix in a process that results in ATP production. Mitochondrial oxidative phosphorylation and ATP production are not perfectly coupled processes, and any protons that reenter the matrix independent of ATP synthase (proton leak) are uncoupled from ATP production. This proton leak occurs as both basal- and inducible-proton leak, which are estimated to be responsible for 20-30% of the resting metabolic rate.
Naturally occurring proton leak can be mimicked with small molecule protonophores that are capable of shuttling protons from the mitochondrial intermembrane space through the mitochondrial inner membrane and into the mitochondrial matrix. Effective proton cycling requires small molecules to permeate the mitochondrial inner membrane in both a neutral and charged state, the latter of which is facilitated by delocalizing the charge into a conjugated system via resonance to ‘effectively mask the charge’. Most well-studied uncouplers contain highly conjugated systems that function as lipophilic weak acids. The ability of potent uncouplers to induce maximum cellular respiration coupled with lack of mitochondrial membrane selectivity may in part contribute to toxicities that have hindered progression to the clinic. A pertinent example is the potent non-mitochondria-selective uncoupler 2,4-dinitrophenol (DNP), that was banned by the FDA due to its narrow therapeutic window. However, mild mitochondrial uncoupling that does not elicit maximum cellular respiration may be a safety feature if suitable potency to suppress weight gain is maintained. Recently, it was shown that the kinase inhibitor Sorafenib (3.4), a treatment for advanced hepatocellular carcinoma, also has mild mitochondrial uncoupling activity and low-dose Sorafenib was demonstrated to be effective in preventing the progression of NASH in rodents and non-human primates. Although the initial in vitro and in vivo results are compelling, Sorafenib’s side effects when administered at higher dose (including fatigue, anorexia, diarrhea, rash and desquamation) may present hurdles for a chronic disease treatment. We postulate that a mitochondrial uncoupler with mild activity that is selective for the mitochondria and lacks off-target actions may have an improved therapeutic window and potential to treat NASH.

We previously reported compound 10b (3.5) as a potent uncoupler that is selective for the mitochondrial membrane. Administration of 3.5 in a streptozotocin (STZ)-induced mouse model
of NASH resulted in a reduction of fibrosis and improvement in NAS.\textsuperscript{60, 61} Building on this work, we sought to address the limitations of 3.5, namely solubility and pharmacokinetic properties. Replacing one aniline moiety with a hydroxyl group provided a new series, with lead compound SHS4121705 (3.6) having vastly improved physiochemical properties, albeit a 23-fold loss of potency.\textsuperscript{62} Nonetheless, 3.6 had great liver localization, lowering all three NASH liver markers (steatosis, inflammation and ballooning) resulting in a 2-point reduction in NAS score. Analogs of SHS4121705 with aliphatic tails, notably the four-carbon butyl and butoxy derivatives, had the greatest potency (EC\textsubscript{50} = 2.1 \textmu M) and resulted in a mild increase in metabolism. In dose-response studies, these compounds maintained the same level of mild uncoupling activity in rat L6 myoblast cells across a broad dose range, without reaching maximum cellular oxygen consumption rate or inducing cytotoxicity.\textsuperscript{62} These results suggest the possibility for a wide therapeutic window. Unfortunately, when dosed in mice, the half-life of these aliphatic analogs was short (< 1 h), presumed to be due to the susceptibility of alkyl chains to \omega-oxidation.\textsuperscript{63} Attempts to address these concerns with common medicinal chemistry approaches such as fluorination resulted in reduced activity.\textsuperscript{64} To capitalize on the protonophoric activity of these compounds while addressing possible metabolic liabilities, we replaced the alkyl chain with an aryl moiety.\textsuperscript{65} In this study, we performed a structure-activity relationship (SAR) investigation of 6-amino-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol derivatives bearing 1,1'-biphenyl aniline moieties as mitochondrial uncouplers. Our investigations indicate that electron withdrawing and donating substitutions on the 1,1'-biphenyl anilines result in compounds with significantly improved pharmacokinetic properties and excellent oral bioavailability. We demonstrate the efficacy of mild mitochondrial uncouplers in a STAM mouse model of NASH resulting in a 1.9-point reduction in NAS score compared to control.
3.4 Results and Discussion

An important property of mitochondrial uncouplers is their ability to cycle between cellular membranes, which would be facilitated by the lipophilic tail of 3.7.\textsuperscript{66,67} We hypothesized that the pharmacokinetic profile (C\textsubscript{max} = 12 µM and t\textsubscript{1/2} = 0.7 h) of 3.7 could be addressed by effectively blocking sites amenable to rapid metabolism. The inclusion of the electron-withdrawing fluorine, trifluoromethoxy, or cyano groups was detrimental to the bioactivity.\textsuperscript{62} We hypothesized that utilization of a phenyl moiety in place of 3.7’s alkyl chain might promote mitochondrial uncoupling while increasing metabolic stability. To test this hypothesis, we synthesized and developed a structure-activity relationship profile of a series of 6-(biphenylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol derivatives.

The synthesis of the pyrazinol derivatives began from common intermediate 5,6-dichloro-[1,2,5]oxadiazolo[3,4-b]pyrazine (3.8), which was prepared as previously described (Scheme 3.1).\textsuperscript{60,68-70} In a one-pot reaction, 3.8 was treated with a sub-stoichiometric equivalent of the necessary aniline followed by aqueous potassium hydroxide to afford the desired 3.9. Additionally, compounds utilizing non-commercially available anilines were obtained via a Suzuki-Miyaura cross-coupling strategy. Therefore, anilines substituted with either a halogen or boronic acid derivative were installed on 3.8, then, the biphenyl 3.9 was synthesized via palladium catalyzed cross-coupling (Scheme 3.1).
Scheme 3.1. Reagents and conditions: (a) i) aniline, Et$_3$N or K$_2$CO$_3$, THF or acetone, rt. or 75 °C; ii) KOH$_{(aq)}$, rt., 2 h, 32-80% yield; (b) aryl-B(OR)$_2$, aryl halide, PdCl$_2$(dppf)•CH$_2$Cl$_2$, Na$_2$CO$_3$, Dioxane/H$_2$O (2:1), 90 °C, 16 h, 1-96% yield; (c) one pot reaction: 4'-fluoro-[1,1'-biphenyl]-4-amine, Et$_3$N, THF, rt.; then NaOMe in MeOH, rt., 1 h, 65% yield.

With 6-(biphenylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol derivatives in hand, we tested their ability to function as mitochondrial uncouplers as a function of oxygen consumption rate (OCR) in L6 rat myoblast cells using an Agilent Seahorse XF analyzer. In this assay, cells are exposed to increasing concentrations of compound and monitored over a 90-minute period for oxygen consumption. Compounds with protonophoric uncoupling activity will elicit increased OCR. BAM15 has been shown to induce maximum mitochondrial respiration and is used to benchmark the activity of tested compounds. Analog activity is reported by integrating the area...
Table 3.1. Oxygen consumption rates of 4-aminobiphenyl derivatives in L6 rat myoblast cells.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;, µM</th>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>-</td>
<td>71%</td>
<td>4.3 ± 0.7</td>
<td>3.9m</td>
<td>-</td>
<td>72%</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>3.9a</td>
<td>-</td>
<td>67%</td>
<td>2.9 ± 0.5</td>
<td>3.9n</td>
<td>-</td>
<td>70%</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>3.9b</td>
<td>(SHS4091862)</td>
<td>73%</td>
<td>2.0 ± 0.3</td>
<td>3.9o</td>
<td>-</td>
<td>8%</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>3.9c</td>
<td>-</td>
<td>25%</td>
<td>4.5 ± 0.7</td>
<td>3.9p</td>
<td>-</td>
<td>6%</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>3.9d</td>
<td>-</td>
<td>23%</td>
<td>6.0 ± 0.3</td>
<td>3.9q</td>
<td>-</td>
<td>58%</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>3.9e</td>
<td>-</td>
<td>8%</td>
<td>4.5 ± 0.4</td>
<td>3.9r</td>
<td>-</td>
<td>24%</td>
<td>9.9 ± 0.8</td>
</tr>
<tr>
<td>3.9f</td>
<td>-</td>
<td>23%</td>
<td>43.3 ± 7.1</td>
<td>3.9s</td>
<td>-</td>
<td>34%</td>
<td>9.3 ± 3.0</td>
</tr>
<tr>
<td>3.9g</td>
<td>-</td>
<td>56%</td>
<td>22.5 ± 5.7</td>
<td>3.9t</td>
<td>-</td>
<td>52%</td>
<td>11.5 ± 1.4</td>
</tr>
<tr>
<td>3.9h</td>
<td>-</td>
<td>47%</td>
<td>6.4 ± 0.9</td>
<td>3.9u</td>
<td>-</td>
<td>46%</td>
<td>67.1 ± 12.6</td>
</tr>
<tr>
<td>3.9i</td>
<td>-</td>
<td>70%</td>
<td>10.4 ± 2.1</td>
<td>3.9v</td>
<td>-</td>
<td>52%</td>
<td>18.9 ± 2.8</td>
</tr>
<tr>
<td>3.9j</td>
<td>-</td>
<td>21%</td>
<td>3.7 ± 0.5</td>
<td>3.9w</td>
<td>-</td>
<td>11%</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>3.9k</td>
<td>-</td>
<td>71%</td>
<td>3.5 ± 0.5</td>
<td>3.9x</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3.9l</td>
<td>-</td>
<td>71%</td>
<td>3.5 ± 0.5</td>
<td>3.9y</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>b</sup> OCR = Oxygen Consumption Rate; EC<sub>50</sub> = Effective Concentration of 50%
under the curve (AUC) and dividing it by the AUC of BAM15 to normalize the data and account for inter-assay variability. The half maximal effective concentration (EC$_{50}$) is measured based on the maximum OCR% of each individual compound. Both parameters (OCR% and EC$_{50}$) are considered in conjunction when evaluating the analogs as a measure of potency and efficacy and are reported in Tables 3.1-2. Using these criteria, mild mitochondrial uncouplers will have OCR between 50-75% of BAM15 activity.

The first analog, **3.9a**, a *para* biphenyl, retained 67% of maximal BAM15 uncoupling capability, which is similar to SHS4121705, but with a 1.5-fold improved potency. Following this encouraging result, an extensive SAR profiling was pursued (Tables 3.1-2). The first series utilized the [1,1'-biphenyl]-4-amine arrangement incorporating a diverse array of electron withdrawing and electron donating groups (Table 3.1). The *para* fluorinated analog **3.9b** resulted in a slight improvement in both potency and efficacy compared to **3.9a**. Replacing the fluorine with trifluoromethoxy (**3.9c**) or difluoromethoxy (**3.9d**) as well as stronger withdrawing trifluoromethyl (**3.9e**) or cayno (**3.9f**) resulted in a reduction of activity. Heterocycles such as 1,3-dioxolane (**3.9g**), 2,2-difluoro-1,3-dioxolane (**3.9h**), thiophene (**3.9i**) or thiophene-2-carbonitrile (**3.9j**) were moderately tolerated, but considerably less effective than **3.9b**. The *meta* substituted difluoro analog **3.9k** was well tolerated with a moderate loss of potency compared to **3.9b**. Interestingly, *meta* substitution with trifluoromethoxy (**3.9l**), trifluoromethyl (**3.9m**) and methoxy (**3.9n**) were well tolerated compared to the *para* regioisomers. Interestingly, fluorination of the aniline ring

| **3.9l** | H | CH$_2$F | OCF$_3$ | 63% | 4.8 ± 0.5 | -- | -- | -- | -- |

*Highest tested concentration is 200 µM.*

*Ratio of integrated area under OCR dose curve above baseline relative to that of BAM15. NA = No Activity.*
(3.9o and 3.9p) resulted in loss of efficacy, although additional fluorine on the distal phenyl ring (3.9q) resulted in moderate activity. Incorporation of polar groups on 3.9b such as hydroxy (3.9r) or methoxy (3.9s) resulted in diminished efficacy and potency. Inclusion of a cyano group in 3.9t and 3.9u resulted in significant loss of activity similar to that observed with 3.9f and 3.9j. Replacement of the biphenyl system with 9H-fluoren-9-one (3.9v), 9,9-dimethyl-9H-fluorene (3.9w) or the nonaromatic (3.9x) resulted in diminished activity. Overall, compounds bearing electron withdrawing groups on the distal ring were the most potent and efficacious while additional fluorination of the aniline ring negatively impacted efficacy.

Next, a series of [1,1'-biphenyl]-3-amine compounds was generated to investigate the effect of the meta biphenyl arrangement (Table 3.2). The meta derivative 3.9y was comparable to 3.9a. Surprisingly, the halogenated meta biphenyl compounds 3.9z and 3.9aa resulted in a >10% increase in OCR activity with comparable potency to 3.9b. The most notable difference between the meta and para biphenyl analogs was the reversal of the regioisomer activity. In the [1,1'-biphenyl]-3-amine series, para substitution was well tolerated with difluoromethoxy (3.9ab), trifluoromethoxy (3.9ac) and trifluoromethyl (3.9ad) having comparable activity to 3.9b whereas the meta-CF₃ derivative 3.9ae resulted in almost complete loss of efficacy. Similarly, multiple fluorines on the distal ring in 3.9af and 3.9ag resulted in reduced efficacy. The methyl substituted 3.9ah produced one of the most active compounds of the series with EC₅₀ = 1.3 ± 0.2. Unlike the para substituted isomers, both 9H-fluoren-9-one (3.9ai) and 9,9-dimethyl-9H-fluorene (3.9aj) had moderate efficacy and potency. The more sterically hindered, a [1,1'-biphenyl]-2-amine derivate (3.9ak) of 3.6 had an almost complete loss of efficacy. Methylation of the phenolic oxygen (3.10), in agreement with our previous work, resulted in complete
Table 3.2. Oxygen consumption rates of 2- or 3-aminobiphenyl derivatives in L6 rat myoblast cells.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR$^b$</th>
<th>EC$_{50}$, µM</th>
<th>Entry</th>
<th>R</th>
<th>% of BAM15 OCR$^b$</th>
<th>EC$_{50}$, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9y</td>
<td></td>
<td>71%</td>
<td>1.9 ± 0.3</td>
<td>3.9af</td>
<td></td>
<td>31%</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>3.9z</td>
<td></td>
<td>85%</td>
<td>1.6 ± 0.2</td>
<td>3.9ag</td>
<td></td>
<td>15%</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>3.9aa</td>
<td></td>
<td>83%</td>
<td>1.4 ± 0.2</td>
<td>3.9ah</td>
<td></td>
<td>69%</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>3.9ab</td>
<td></td>
<td>60%</td>
<td>2.0 ± 0.2</td>
<td>3.9ai</td>
<td></td>
<td>63%</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>3.9ac</td>
<td></td>
<td>60%</td>
<td>3.3 ± 0.4</td>
<td>3.9aj</td>
<td></td>
<td>49%</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>3.9ad</td>
<td></td>
<td>74%</td>
<td>2.6 ± 0.4</td>
<td>3.9ak</td>
<td></td>
<td>3%</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>3.9ae</td>
<td></td>
<td>9%</td>
<td>1.7 ± 0.4</td>
<td>3.10</td>
<td></td>
<td>--</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$Highest tested concentration is 200 µM.

$^b$Ratio of integrated area under OCR dose curve above baseline relative to that of BAM15. NA = No Activity.

abrogation of the compounds’ activity suggesting the role of the hydroxyl group as the protonophore.$^{62}$

Based on the OCR assay, several compounds had greater than a two-fold increase in potency, compared to our previous findings, while maintaining desirable physiochemical properties. Importantly, [1,1'-biphenyl]-amine analogs demonstrated self-limiting activity allowing for mild mitochondrial uncoupling to be maintained at high dose, without achieving maximum oxygen rate consumption (Figure 3.2). This unique confluence of potency and stable mild-uncoupling activity is a departure from previously reported uncouplers.
Figure 3.2. Oxygen consumption rate demonstrating the mild-uncoupling activity of [1,1'-biphenyl]-amine compounds relative to BAM15. Data are averaged over three separate experiments. Error bars represent standard error of mean.

To evaluate the pharmacokinetic properties, compounds with %BAM15 OCR > 65% with no negative slope over the concentration range and < 5 μM potency were selected, and mice were dosed with 10 mg/kg per oral. As shown in Table 3.3, inclusion of [1,1'-biphenyl]-amines resulted in good to excellent oral bioavailability with a broad range of half-life (0.53 to > 24h). Among the most promising, 3.9b (SHS4091862) resulted in a peak plasma concentration of ~46 μM with a half-life of 4.7 hours following per oral administration in mice (Figure 3.3). The mild uncoupling activity combined with favorable pharmacokinetic properties suggested 3.9b would be a suitable candidate for in vivo efficacy studies against NASH.

In vivo efficacy was investigated using the Stelic animal model (STAM) of NASH with Resmetirom, a leading compound currently in Phase III clinical trials for NASH, as the positive control.27-29 Mice were administered either 3.9b (60 mg/kg) or Resmetirom (3 mg/kg) admixed in high-fat diet over the course of 28 days. Food consumption was monitored during the treatment period; however, mice were group-housed 4 per cage (2 cages each treatment group) and, thus,
**Table 3.3.** Pharmacokinetic profile of select analogs in mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9a</td>
<td><img src="image" alt="Structure" /></td>
<td>3.8</td>
<td>0.53</td>
</tr>
<tr>
<td>3.9b</td>
<td><img src="image" alt="Structure" /></td>
<td>45.8</td>
<td>4.7</td>
</tr>
<tr>
<td>3.9m</td>
<td><img src="image" alt="Structure" /></td>
<td>90.5</td>
<td>&gt; 24</td>
</tr>
<tr>
<td>3.9n</td>
<td><img src="image" alt="Structure" /></td>
<td>2.8</td>
<td>1.1</td>
</tr>
<tr>
<td>3.9y</td>
<td><img src="image" alt="Structure" /></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3.9ad</td>
<td><img src="image" alt="Structure" /></td>
<td>32.4</td>
<td>22.9</td>
</tr>
<tr>
<td>3.9ah</td>
<td><img src="image" alt="Structure" /></td>
<td>1.95</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* Compounds were administered at 10 mg/kg body weight by oral gavage to Swiss Albino male mice. Plasma samples collected over 8-24 h were analyzed by LC-MS/MS. C<sub>max</sub> = maximal plasma concentration. t<sub>1/2</sub> = half-life.

Statistics could not be performed. Nonetheless, both 3.9b and Resmetirom had negligible effect on food consumption compared to vehicle controls (mice ate 5.5% and 2.6% less food than controls, respectively). As shown in Figure 3.4A-B, liver fibrosis score based on picrosirius red stained area was not statistically affected by either 3.9b or Resmetirom; however, the vehicle control score (0.93 ± 0.26%) was greater than both 3.9b (0.76 ± 0.16%) and positive control Resmetirom (0.69 ± 0.17%). Treatment with 3.9b resulted in a 1.9-point improvement in NAS score compared to...
Figure 3.3. Pharmacokinetic study of 3.9b. 3.9b was administered at 10 mg/kg body weight to Swiss Albino mice by oral gavage and plasma samples were collected at the time points shown and analyzed by LC–MS/MS. n=3 male mice per group.

vehicle control mice (Figure 3.4C, 2.75 ± 0.7 compared to 4.63 ± 0.7 in vehicle), due to a lower score in all three liver NAS markers: steatosis (0.1 ± 0.4), inflammation (2.4 ± 0.5), ballooning (0.3 ± 0.5) (Figure 3.4D-F). Mice treated with 3.9b resulted in an almost complete decrease in steatosis and ballooning with a moderate decrease in inflammation.

The impact on whole body physiology following 3.9b treatment was also investigated. There was no change in body weight or liver-to-body weight ratio compared to vehicle control following treatment with 3.9b, while Resmetirom decreased liver-to-body weight ratio (Figure 3.5A-C). Both 3.9b and Resmetirom decreased liver triglycerides by 37% and 62% compared to control, respectively (Figure 3.5D). 3.9b did not alter plasma triglycerides, cholesterol or blood glucose levels, while Resmetirom lowered cholesterol (Figure 3.5E-G). 3.9b treatment resulted in no change to alanine aminotransferase (ALT) or aspartate aminotransferase (AST) compared to vehicle control (Figure 3.5H-I). In contrast, Resmetirom treatment increased ALT levels, a known
Figure 3.4. Histological results from liver tissue of STAM mice. Representative images of hematoxylin and eosin and Sirius Red staining of fixed tissue (A). Fibrosis was measured by quantification of Sirius Red-positive area (B). NAFLD activity scores (C) were calculated from pathologist scoring of steatosis (D), inflammation (E), and ballooning (F). * indicates p< 0.05. Statistical significance was assessed by one-way ANOVA for normally distributed data (D) and Kruskal–Wallis test for nonparametric data (B, C, E, F). n=7-8 male mice per group.
**Figure 3.5.** Physiological parameters of STAM mice. 3.9b decreased body weight (A), with a slight increase in liver weight (B), resulting in no change to liver-to-body weight ratio (C). Both drug treatments decreased liver triglycerides (D). 3.9b did not change plasma triglyceride (E) or cholesterol (F) levels or whole blood glucose (G). 3.9b had no effect, while Resmetirom treatment resulted in an increase in ALT levels (H). Both drug treatments had no effect on plasma AST (I). * indicates p < 0.05. Statistical significance was assessed by one-way ANOVA for normally distributed data (A-D, H) and Kruskal–Wallis test for nonparametric data (E-G, I). n = 7-8 male mice per group.

marker of liver damage (Figure 3.5H). 74, 75 These results suggest that mild mitochondrial uncouplers have potential for the development of NASH therapeutics.

### 3.5 Conclusions

Mild mitochondrial uncoupling that increases nutrient metabolism without triggering maximal mitochondrial respiration rate may prove to be an attractive therapeutic approach against metabolic diseases. 76-79 In this study, we sought to develop potent mitochondrial uncouplers with this property for diseases requiring regimes of long term drug exposure. The structure-activity relationship of 6-(biphenylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol derivatives resulted in a new class of mild mitochondrial uncouplers with in vivo efficacy in a mouse model of NASH. The
derivatization of the aniline moiety while maintaining the [1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol core expanded the chemical space and increased potency.\textsuperscript{62} We demonstrated that 1,1'-biphenyl aniline derivatives resulted in excellent oral bioavailability. Our investigations revealed that both electron donating and withdrawing groups were tolerated while eliciting mild-uncoupling activity. Electron withdrawing groups in the distal aryl ring were among the most potent and efficacious compound with favorable pharmacokinetic properties. The combination of excellent oral bioavailability coupled with mild mitochondrial uncoupling activity encouraged the advancement into \textit{in vivo} efficacy studies of 3.9b. In the STAM mouse model of NASH, 3.9b decreased fibrosis score compared to vehicle control mice with a near-complete normalization of steatosis and ballooning. 3.9b treated mice had a 1.9-point improvement in NAS score compared to vehicle control without altering body weight or food intake. In contrast, Resmetirom did not result in a statistically significant decrease in NAS score compared to vehicle control, and also raised ALT levels. Based on this benchmark, our results suggest that these compounds represent a potential therapeutic avenue for the treatment of NASH.

3.6 References


43. Tainter, M. L.; Stockton, A. B.; Cutting, W. C., Use Of Dinitrophenol In Obesity And Related Conditions: A Progress Report. JAMA 1933, 101, 1472-1475.


Chapter 4: Experimental Section

4.1 General Experimental Methods

All solvents used were either dried with a PureSolv solvent purification system prior to use or purchased in anhydrous form. All chemical reagents were purchased from commercial sources and used without further purification. Thin layer chromatography (TLC) was performed on aluminum-backed silica gel, 200 µm, F254. Column chromatography was performed on flash grade silica gel, 40-63 µm, with a Teledyne ISCO Combiflash Rf purification system.

4.1.1 Instrumentation

NMR spectra were recorded using an Agilent 400-MR 400 MHz, a Varian Inova 400 MHz or Bruker Avance II 500. $^1$H NMR chemical shifts are reported in ppm with the solvent resonance as an internal standard ((CD$_3$)$_2$SO: 2.50 ppm; (CD$_3$)$_2$CO: 2.05 ppm; CDCl$_3$: 7.26 ppm). $^{13}$C NMR chemical shifts are reported in ppm with the solvent resonance as the internal standard ((CD$_3$)$_2$SO: 39.52 ppm; (CD$_3$)$_2$CO: 29.84/206.26 ppm; CDCl$_3$: 77.16 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), and integration. HPLC and high-resolution mass spectroscopy (HRMS) was performed on Thermo Electron TSQ triple quadrupole mass spectrometer equipped with an ESI source. HPLC condition 1: Phenomenex LUNA column (150 mm x 2.0 mm, 5 µm, C18) using a solvent system of 1% formic acid in H$_2$O (mobile phase A) and 1% formic acid in acetonitrile (mobile phase B) on an Agilent 1100 binary pump with a gradient of 50-95% mobile phase A→B at a flow rate of 0.2 mL/min. HPLC condition 2: Phenomenex Luna (150 mm x 4.5 mm, 5 µm, C18) using a solvent system of 0.1% TFA in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B) on an Agilent 1200 binary pump with a gradient of 5-95% mobile phase A→B at a flow rate of 1.5 mL/min. Unless otherwise noted, HPLC condition 1 was used.
All compounds tested in biological assays are >95% pure by HPLC analyses unless noted otherwise.

4.1.2 Oxygen Consumption Rate Seahorse Assay.

Oxygen consumption rate (OCR) was measured using an Agilent Seahorse XF24 or XFe96 Analyzer (Agilent Technologies, Santa Clara, CA). L6 myoblasts were seeded in a Seahorse 24 or 96-well tissue culture plate at a density of $3.5 \times 10^4$ cells/well. The cells were then allowed to adhere overnight. Prior to the assay, the media was changed to unbuffered DMEM containing pyruvate and glutamine (Gibco #12800-017, pH = 7.4 at 37 °C) and the cells were equilibrated for 1hr at 37 °C without CO$_2$. Compounds were injected during the assay and OCR was measured using 2 min measurement periods. Cells were treated with a single drug concentration per well and measured over a 90 min period. 2 wells were used per condition and, where applicable, results from multiple plates were averaged together. The first three measurements after injection for each concentration were averaged to produce a dose curve. EC$_{50}$ values were calculated using GraphPad Prism’s non-linear regression built-in equation, $Y=\text{Bottom} + (X^{\text{Hillslope}})*(\text{Top}-\text{Bottom})/(X^{\text{Hillslope}} + \text{EC50}^{\text{Hillslope}})$, with the Bottom constrained to the 100% baseline. Area under curve (AUC) values were also calculated using the same software.

4.2 Synthetic Procedures and Characterization of Compounds for Chapter 2

4.2.1 Acute Dose Experiments.

Acute dose experiments were performed at UNSW and approved by the UNSW Animal Care and Ethics Committee (Project Approval 17-66B). Mice were purchased from Australian BioResources (Moss Vale, NSW, Australia). Mice were housed at 22 °C in a light/dark cycle of 12h. Unless otherwise stated, mice were provided with *ad libitum* access to water and standard chow diet (Gordons Specialty Feeds, NSW, Australia).
To measure pharmacokinetics for **SHS4121705 (2.12i), 2.12ah, 2.12az, 2.12bh, 2.12bj** and **2.12ce**, compounds were administered as a bolus to 9-12 week-old male C57BL/6 mice by oral gavage at a dose of 10 mg/kg body weight. Compounds were delivered in a mixture containing 90-91% (v/v) methylcellulose (Sigma, M0512), 2-3% (v/v) Tween 80 (Sigma, P6474), and 6-8% (v/v) DMSO (Sigma, D5879). Blood samples were collected from the tail tip at the time points shown in heparinized capillary tubes. Plasma was collected by centrifugation at 2000 g for 10 minutes. Samples were processed for LC-MS/MS by extraction in 100 µL acetonitrile:methanol (9:1) for 7 µL plasma, followed by centrifugation at 18 000 g for 10 min and collection of the supernatant for analysis. Standards were prepared by spiking in known amounts (1-100 ng) of compound into plasma from untreated mice and processed the same way. LC–MS/MS was performed on a Shimadzu Prominence LCMS-8030 (Shimadzu, Japan). Chromatographic separation was achieved using an ACUITY UPLC BEH, C18 column (Waters, WT186002350, USA). Mobile phase A consisted of 0.1% v/v formic acid in HPLC grade water. Mobile phase B consisted of 0.1% v/v formic acid in acetonitrile. The analyte was eluted with a gradient of 5–80% mobile phase B at a flow rate of 0.4 mL/min with 10 µL injection volume electrosprayed into the mass spectrometer. ESI was performed in negative mode. Identification was achieved by the following m/z transitions and retention times: 313 → 201, 284 at 5.3 min for **SHS4121705 (2.12i)**; 301 → 243, 215 at 4.7 min for **2.12ah**; 279 → 167, 125 at 4.8 min for **2.12az**; 285 → 174 at 5 min for **2.12bh**; 284 → 173 at 4.6 min for **2.12bj**. Quantification was determined by measuring peak areas using LabSolutions software on the instrument. Concentration of test samples was interpolated from a standard curve derived from the integrated intensity values of standards.

Pharmacokinetics analysis for compound **2.12ce** was performed by GVK Biosciences (Hyderabad, India). Swiss Albino mice were administered 10 mg/kg **2.12ce** by oral gavage in a triturated
formulation with Tween 80 and 0.5% (w/v) methylcellulose. Blood samples were collected at time points indicated in the figure, with three replicates per time point. Plasma was separated by centrifugation. 50 µL of plasma was precipitated with 200 µL of acetonitrile containing internal standard of Telmisartan at 200 ng/mL. Samples were vortexed for 5 min at 850 rpm and centrifuged at 4000 rpm for 5 min at 4 °C. From this, 110 µL of supernatant was diluted with 150 µL of methanol:water (1:1, v/v). Calibration standards were prepared using 2.0 µL of calibration curve standard added to 48 µL blank matrix, which was processed in the same way as 50 µL of sample plasma. Liquid chromatography tandem mass spectrometry was performed on a 5500 QTRAP (SCIEX, USA). Chromatographic separation was achieved using a Kinetex Evo, C18, 50*4.6 mm, 5 microns. Mobile phase A consisted of 10 mM ammonium acetate with 0.1% v/v formic acid in MilliQ water. Mobile phase B consisted of acetonitrile:methanol (50:50). The analyte was eluted with a gradient of 5-95% mobile phase B at a flow rate of 1 mL/minute with 15 µL injection volume. Electrospray Ionization (ESI) was performed in negative mode. Transitions of m/z 286 → 125, and 1.64-minute retention time was used to identify 2.12ce, and 513.2 → 287.1, and 1.59-minute retention time was used to identify Telmisartan as an internal standard. Concentration of test samples was interpolated from a standard curve derived from the intensity values of standards (1-1000 ng/mL). Animal studies performed by GVK Biosciences were approved by its Institutional Animal Ethics Committee.

To measure body temperature, mice were similarly administered an oral bolus of SHS4121705 (2.12i), except that doses ranged from 0 (vehicle) to 50 mg/kg body weight. Core body temperature was measured with a rectal probe thermometer (Braintree, TW2-107) at the time points shown.
To measure tissue distribution, mice were similarly orally administered a bolus of SHS4121705 (2.12i) at 10 mg/kg body weight. Mice were euthanized by cervical dislocation after 1 h, and tissues were dissected, rinsed in PBS, and snap-frozen in liquid nitrogen. To process the tissues for LC-MS/MS analysis, frozen tissues were powdered in liquid nitrogen using a tissue pulverizer (Cellcrusher, USA), then homogenized with a motorized pellet pestle homogenizer. Homogenate was centrifuged (10 000g for 10 min) and supernatant collected. Samples were processed and analyzed as described for plasma samples above.

4.2.2 Mouse Study.

The STAM mouse model was performed by SMC Laboratories, Inc (Tokyo, Japan). Compound SHS4121705 (2.12i) was administered in diet by premixing high-fat diet (HFD, 57 kcal% fat, Cat# HFD32, CLEA Japan Inc., Japan) at SMC Laboratories. Telmisartan (Micardis®) was purchased from Boehringer Ingelheim GmbH (Germany) and dissolved in pure water. NASH was induced in 24 male mice by a single subcutaneous injection of 200 μg streptozotocin (Sigma-Aldrich, USA) solution 2 days after birth and feeding with HFD after 4 weeks of age. From 6 weeks of age, SHS4121705 (2.12i) was administered orally by feeding with HFD to achieve a dose of 25 mg/kg/d, and Telmisartan was administered orally in a volume of 10 mL/kg to achieve a dose of 10 mg/kg/d. The viability, clinical signs and behavior were monitored daily. Body weight was recorded before the treatment. Mice were observed for significant clinical signs of toxicity, moribundity and mortality approximately 60 minutes after each administration. The animals were sacrificed at 9 weeks of age (Day 21) by exsanguination through direct cardiac puncture under isoflurane anesthesia.

Non-fasting blood glucose was measured in whole blood using Stat Strip glucose meter (Nipro Corporation, Japan). Readings greater than 900 mg/dL (one mouse in Telmisartan group)
were recorded as 900 mg/dL. For plasma biochemistry, non-fasting blood was collected in polypropylene tubes with anticoagulant (Novo-Heparin, Mochida Pharmaceutical Co. Ltd., Japan) and centrifuged at 1,000 xg for 15 minutes at 4 °C. The supernatant was collected and stored at -80 °C until use. Plasma ALT level was measured by FUJI Dri-Chem 7000 (Fujifilm, Japan).

For liver samples, the left lateral lobe was collected and cut into 6 pieces. Two pieces of left lateral lobe, left and right medial lobes, and caudate lobe were snap frozen in liquid nitrogen and stored at -80 °C. The other 2 pieces of left lateral lobe were fixed in Bouin’s solution and then embedded in paraffin. Samples were stored at room temperature for histology. Samples were stored at -80 °C. The right lobe was snap frozen in liquid nitrogen and stored at -80 °C for liver biochemistry.

Liver total lipid-extracts were obtained by Folch’s method. Liver samples were homogenized in chloroform-methanol (2:1, v/v) and incubated overnight at room temperature. After washing with chloroform-methanol-water (8:4:3, v/v/v), the extracts were evaporated to dryness, and dissolved in isopropanol. Liver triglyceride content was measured by Triglyceride E-test (Wako Pure Chemical Industries, Ltd., Japan).

For histopathology staining, sections were cut from paraffin blocks of liver tissue prefixed in Bouin’s solution and stained with Lillie-Mayer’s Hematoxylin (Muto Pure Chemicals Co., Ltd., Japan) and eosin solution (Wako Pure Chemical Industries). NAFLD Activity score (NAS) was calculated according to the criteria of Kleiner.93 To visualize collagen deposition, Bouin’s fixed liver sections were stained using picro-Sirius red solution (Waldeck, Germany). For quantitative analysis of fibrosis areas, bright field images of Sirius red-stained sections were captured around the central vein using a digital camera (DFC295; Leica, Germany) at 200-fold magnification, and
the positive areas in 5 fields/section were measured using ImageJ software (National Institute of Health, USA).

The animals were maintained in a SPF facility under controlled conditions of temperature (23 ± 2 °C), humidity (45 ± 10%), lighting (12-hour artificial light and dark cycles; light from 8:00 to 20:00) and air exchange. A high pressure was maintained in the experimental room to prevent contamination of the facility. All animals used in the study were housed and cared for in accordance with the Japanese Pharmacological Society Guidelines for Animal Use. This STAM experiment using SHS4121705 (2.12i) was conducted as part of a study using the same control groups (vehicle and Termisartan) as previously published.78

4.2.3 Plasma Biochemistry.

Plasma triglyceride was measured by a colorimetric assay through reaction with GPO reagent (Pointe Scientific T7532) according to the manufacturer’s protocol. A standard curve was constructed using serial dilutions of glycerol standard (Sigma G7793). In brief, samples were incubated with GPO at 37 °C for 5–20 min until absorbance values at 500 nm had stabilized. Sample plasma triglyceride concentrations were determined through interpolation from the standard curve. Plasma cholesterol was measured by a colorimetric assay using Infinity cholesterol liquid stable reagent (ThermoFisher TR13421) according to the manufacturer’s protocol. A standard curve was constructed using serial dilutions of cholesterol standard (Pointe Scientific C7509). In brief, samples were incubated with Infinity cholesterol reagent at 37 °C for 5–20 min until absorbance values at 500 nm had stabilized. Absorbance at 660 nm was subtracted from absorbance at 500 nm to correct for background. Sample plasma cholesterol concentrations were determined through interpolation from the standard curve. Plasma AST was measured using Infinity AST (GOT) liquid stable reagent (ThermoFisher TR70121) according to the
manufacturer’s protocol. In brief, samples were incubated with the Infinity AST reagent at 37 °C and absorbance was measured at 340 nm every minute for 20 min. Activity in U/L was calculated by multiplying the change in absorbance per minute by a correction factor, which was defined as total volume of reaction divided by the product of the molar absorption coefficient of NADH, the sample volume added, and the path length of absorption.

4.2.4 Statistical Analysis.

All data are presented as the mean ± standard error of the mean (SEM). Statistical testing was carried out using Prism (v.8.1.2; GraphPad Software), where the threshold for significance was designated as p<0.05, compared to vehicle. For normally distributed data, differences between groups were examined using a One-Way Analysis of Variance (ANOVA) with Dunnett’s post-hoc test for multiple comparisons. For non-parametric data, the Kruskal-Wallis test was conducted with Dunn’s post-hoc test for multiple comparisons.

4.2.5 General Synthetic Procedures.

**General Procedure 2A:** In a screw-cap vial or round-bottom flask, the requisite amine (0.70 – 0.98 mmol) was added to a stirring mixture of dichloro 2.11 (0.200 g, 1.05 mmol) in anhydrous THF (or acetone where indicated) (0.1 – 0.2 M). Then, Et₃N (1.1 mmol or 2.2 mmol when an amine salt is used) was added and the resulting dark mixture was stirred at room temperature (unless otherwise indicated) for 2 – 20 h. The reaction mixture was diluted with an aqueous solution of KOH (6 equiv.) and stirring at room temperature was continued for 0.5 – 2 h. The mixture was acidified with 1 M aqueous HCl and extracted with EtOAc. The organic layer was washed with brine, dried (Na₂SO₄), and concentrated to a residue. The residue was purified by chromatography on SiO₂ using a MeOH/CH₂Cl₂ or EtOAc/hexanes solvent system to yield the desired product. When additional purification was needed, the solid was dissolved in a minimal
amount of hot acetone, allowed to cool to room temperature, and precipitated by the addition of hexanes. The precipitate was filtered, rinsed with hexanes, and collected to yield the desired product 2.12.

**General Procedure 2B:** To a round-bottom flask containing a stir bar was added dichloro 2.11 (1.05 mmol) which was diluted with anhydrous THF (4 mL), followed by the amine (0.838 mmol, 0.8 equiv.). The resulting solution was stirred at 50 °C for 16 h. KOH (6.28 mmol, 6.0 equiv.) in H₂O (8 mL) and THF (2 mL) was added and stirring was continued for an additional 1 h. The mixture was then acidified with 1 M aqueous HCl and extracted with EtOAc. The organic layer was washed with brine, dried (Na₂SO₄), and concentrated to a solid. The solid was purified by chromatography on SiO₂ using a MeOH/CH₂Cl₂ solvent system to yield the desired compound 2.12.

**General Procedure 2C:** To anhydrous THF (25 mL) was added sodium hydride (1.5 equiv., 60% dispersion) and allowed to stir for 5 min. Benzyl alcohol (1.0 equiv.) in anhydrous THF (15 mL) was added dropwise over a minute and allowed to stir for 30 min. 2,3-Dichloropyrazine (2.14) (1.0 equiv.) in THF (10 mL) was added and the reaction was allowed to stir at room temperature for 2 h (monitored by TLC). Upon completion, the reaction was quenched slowly with isopropanol. The solvent was evaporated under reduced pressure and the crude product was purified by chromatography on SiO₂ using EtOAc/hexanes to produce intermediate 2.15.

A vial was charged with Pd₂dba₃ (0.05 equiv.), Xantphos (0.10 equiv.), 2-(benzyloxy)-3-chloropyrazine (2.15) (1.00 equiv.), and K₂CO₃ (2.00 equiv.). The vial was sealed with a septum and then evacuated and backfilled with argon (3x). Deoxygenated anhydrous dioxane (4 mL) was added through the septum, followed by the requisite aryl amine (1.05 equiv.). The resulting mixture was stirred at 110 °C for 16 h and then allowed to cool to room temperature. The solid
material was filtered through Celite and washed with EtOAc. The filtrate was concentrated under reduced pressure in a round-bottom flask and diluted with methanol (4 mL). The flask was flushed with nitrogen for 5 min. 10% Pd/C (0.05 equiv.) was added and the mixture was stirred under an atmosphere of hydrogen gas (1 atm, balloon) for 1.5 h at room temperature. The reaction was then filtered through a Celite plug washing with EtOAc, concentrated under reduced pressure, and the crude product was purified by chromatography on SiO$_2$ using EtOAc/hexanes to yield the desired compound 2.16.

4.2.6 Characterizations.

6-((2-Fluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12a). Synthesized by procedure 2A to yield 2.12a in 8% as a pale yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.18 (br s, 1 H), 9.23 (s, 1 H), 8.51 (t, $J = 8.2$ Hz, 1 H), 7.35-7.27 (m, 3 H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -129.33 to -129.40 (m, 1 F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) $\delta$ 155.0 (d, $J_{CF} = 245.7$ Hz), 153.5, 151.4, 150.2, 145.1, 127.2 (d, $J_{CF} = 7.7$ Hz), 126.3 (d, $J_{CF} = 10.4$ Hz), 125.6 (d, $J_{CF} = 3.9$ Hz), 124.1, 116.2 (d, $J_{CF} = 19.5$ Hz); HRMS (ESI) $m/z$ calc’d. for C$_{10}$H$_5$FN$_5$O$_2$ (M-H)$^-$ 246.0433, found 246.0438.

6-((3-Fluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12b). Synthesized by procedure 2A to yield 2.12b in 10% as a pale yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.09 (br s, 1 H), 9.64 (s, 1 H), 8.13 (dt, $J = 11.6$, 2.4 Hz, 1 H), 7.90 (dd, $J = 8.2$, 2.0 Hz, 1 H), 7.48 (q, $J = 8.0$ Hz, 1 H), 6.99 (dt, $J = 8.4$, 2.5 Hz, 1 H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -113.15 to -113.22 (m, 1 F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) $\delta$ 163.6 (d, $J_{CF} = 242.4$ Hz), 153.4, 151.4, 150.2, 145.1, 140.4 (d, $J_{CF} = 11.1$ Hz), 131.3 (d, $J_{CF} = 9.4$ Hz), 117.9 (d, $J_{CF} = 3.1$ Hz), 112.3 (d, $J_{CF} = 21.3$ Hz), 109.0 (d, $J_{CF} = 26.9$ Hz); HRMS (ESI) $m/z$ calc’d. for C$_{10}$H$_5$FN$_5$O$_2$ (M-H)$^-$ 246.0433, found 246.0440.
6-((4-Fluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12c). Synthesized by procedure 2B to yield 2.12c in 44% as a yellow solid (HPLC condition 2): \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 11.69 (s, 1H), 9.58 (s, 1H), 8.19 – 8.11 (m, 2H), 7.28 – 7.19 (m, 2H); \(^1\)\(^9\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -118.44 – -118.56 (m, 1F); \(^1\)\(^3\)C NMR (101 MHz, Acetone-\(d_6\)) \(\delta\) 160.6 (d, \(J\)\(_{CF}\) = 243.0 Hz), 153.6, 151.2, 150.4, 145.1, 135.0 (d, \(J\)\(_{CF}\) = 2.7 Hz), 124.2 (d, \(J\) = 8.0 Hz), 116.3 (d, \(J\) = 22.8 Hz); HRMS (ESI\(^+\)) \(m/z\) calc’d for C\(_{10}\)H\(_7\)FN\(_5\)O\(_2\) (M+H)\(^+\) 248.0578, found 248.0591.

6-((2-(Trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12d). Synthesized by procedure 2B to yield 2.12d in 5% as a yellow solid: \(^1\)H NMR (400 MHz, Acetone-\(d_6\)) \(\delta\) 12.1 (s, 1H), 9.36 (s, 1H), 8.55 (d, \(J\) = 8.1 Hz, 1H), 7.88 – 7.79 (m, 2H), 7.50 (t, \(J\) = 7.7, 1.0 Hz, 1H); \(^1\)\(^9\)F NMR (Acetone-\(d_6\), 376 MHz) \(\delta\) rotamers -61.19 (s), -61.21 (s); HRMS (ESI\(^+\)) \(m/z\) calc’d for C\(_{11}\)H\(_7\)F\(_3\)N\(_5\)O\(_2\) (M+H)\(^+\) 298.0546, found 298.0538.

6-((3-(Trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12e). Synthesized by procedure 2B to yield 2.12e in 79% as a yellow solid (HPLC condition 2): \(^1\)H NMR (Acetone-\(d_6\)) \(\delta\) 12.1 (s, 1H), 9.36 (s, 1H), 8.55 (d, \(J\) = 8.1 Hz, 1H), 7.88 – 7.79 (m, 2H), 7.50 (t, \(J\) = 7.7, 1.0 Hz, 1H); \(^1\)\(^9\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) rotamers -61.19 (s), -61.21 (s); HRMS (ESI\(^+\)) \(m/z\) calc’d for C\(_{11}\)H\(_7\)F\(_3\)N\(_5\)O\(_2\) (M+H)\(^+\) 298.0546, found 298.0538.

6-((4-(Trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12f). Synthesized by procedure 2A to yield 2.12f in 66% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.11 (br s, 1 H), 9.77 (s, 1 H), 8.37 (d, \(J\) = 8.3 Hz, 2 H), 7.80 (d, \(J\) = 8.3 Hz, 2 H); \(^1\)\(^9\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -62.59 (s, 3 F); \(^1\)\(^3\)C NMR ((CD\(_3\))\(_2\)CO, 126 MHz) \(\delta\) 153.4, 151.6,
150.2, 145.1, 142.1, 127.0 (q, $J_{CF} = 3.8$ Hz), 126.7 (q, $J_{CF} = 32.6$ Hz), 125.3 (q, $J_{CF} = 270.8$ Hz), 122.2; HRMS (ESI) $m/z$ calc’d. for C$_{11}$H$_5$F$_3$N$_5$O$_2$ (M-H)$^-$ 296.0401, found 296.0419.

6-((2-(Trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12g).

Synthesized by procedure 2A to yield 2.12g in 27% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.25 (br s, 1H), 9.34 (s, 1H), 8.75 (d, $J = 8.1$, 1H), 7.57 – 7.50 (m, 2H), 7.38 (t, $J = 7.5$ Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ rota... 58.522 (s); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) $\delta$ 153.7, 151.3, 150.5, 145.2, 140.3, 130.8, 128.9, 126.8, 123.7, 121.9, 121.6 (q, $J_{CF} = 258.0$ Hz); HRMS (ESI$^+$) $m/z$ calc’d. for C$_{11}$H$_7$F$_3$N$_5$O$_3$ (M+H)$^+$ 314.0496, found 314.0474.

6-((3-(Trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12h).

Synthesized by procedure 2A to yield 2.12h in 64% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.07 (br s, 1H), 9.67 (s, 1H), 8.27 – 8.24 (m, 2H), 7.47 (d, $J = 9.1$ Hz, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -58.79 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) $\delta$ 153.5, 151.3, 150.2, 150.1 (q, $J_{CF} = 3.8$ Hz), 145.1, 140.3, 131.3, 121.5 (q, $J_{CF} = 255.9$ Hz), 120.7, 117.8, 114.6; HRMS (ESI$^-$) $m/z$ calc’d. for C$_{11}$H$_5$F$_3$N$_5$O$_3$ (M-H)$^-$ 312.0350, found 312.0372.

6-((4-(Trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (SHS4121705, 2.12i). Synthesized by procedure 2A to yield SHS4121705 (2.12i) in 75% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.07 (br s, 1H), 9.67 (s, 1H), 8.27 – 8.24 (m, 2H), 7.47 (d, $J = 9.1$ Hz, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -58.79 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) $\delta$ 153.5, 151.3, 150.3, 146.4 (q, $J_{CF} = 2.0$ Hz), 145.1, 137.7, 123.7, 122.5, 121.5 (q, $J_{CF} = 255.3$ Hz); HRMS (ESI$^+$) $m/z$ calc’d. for C$_{11}$H$_5$F$_3$N$_5$O$_3$ (M-H)$^+$ 312.0350, found 312.0369.
6-((2-Chlorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12j). Synthesized by procedure 2A to yield 2.12j in 24% as a yellow solid: ¹H NMR ((CD₃)₂CO, 400 MHz) δ 12.26 (br s, 1H), 9.51 (br s, 1H), 8.78 – 8.75 (m, 1H), 7.62 – 7.59 (m, 1H), 7.54 – 7.50 (m, 1H), 7.30 – 7.26 (m, 1H); ¹³C NMR ((CD₃)₂CO, 126 MHz) δ *rotamers 153.7, 151.2, 150.2, 145.1, 134.8, 130.4, 129.0, 127.0, 126.9*, 125.3, 123.1, 123.0*; HRMS (ESI⁻) m/z calc’d for C₁₀H₅ClN₅O₂ (M⁻H)⁻ 262.0137, found 262.0160.

6-((4-Chlorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12k). Synthesized by procedure 2A to yield 2.12k in 59% as a yellow solid: ¹H NMR ((CD₃)₂CO, 500 MHz) δ 12.06 (br s, 1H), 9.61 (br s, 1H), 8.18 – 8.15 (m, 2H), 7.50 – 7.47 (m, 2H); ¹³C NMR ((CD₃)₂CO, 126 MHz) δ 153.5, 151.3, 150.3, 145.1, 137.6, 130.4, 129.7, 123.7; HRMS (ES⁺) m/z calc’d for C₁₀H₇ClN₅O₂ (M⁺H)⁺ 264.0283, found 264.0289.

6-((4-(Difluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12l). Synthesized by procedure 2A to yield 2.12l in 72% as a pale yellow solid: ¹H NMR ((CD₃)₂CO, 500 MHz) δ 12.05 (br s, 1H), 9.60 (s, 1H), 8.19 – 8.15 (m, 2H), 7.31 – 7.27 (m, 2H), 7.01 (t, J_HF = 74.3 Hz, 1H); ¹⁹F NMR ((CD₃)₂CO, 376 MHz) δ -82.64 (d, J = 74.1 Hz, 2F); ¹³C NMR ((CD₃)₂CO, 126 MHz) δ 153.5, 151.2, 150.4, 149.1 (t, J_CF = 3.2 Hz), 145.1, 136.0, 123.8, 120.5, 117.5 (t, J_CF = 257.4 Hz); HRMS (ES⁺) m/z calc’d for C₁₁H₈F₂N₃O₃ (M+H)⁺ 296.0590, found 296.0594.

6-((4-(1,1,2,2-Tetrafluoroethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12m). Synthesized by procedure 2A to yield 2.12m in 70% as a yellow solid: ¹H NMR ((CD₃)₂CO, 500 MHz) δ 12.07 (br s, 1H), 9.61 (br s, 1H), 8.19 – 8.15 (m, 2H), 7.40 – 7.38 (m, 2H), 6.53 (tt, J_HF = 52.5, 3.0 Hz, 1H); ¹⁹F NMR ((CD₃)₂CO, 376 MHz) δ -89.10 (td, J = 5.5, 2.7 Hz, 2F), -138.63 (dt, J = 52.4, 5.8 Hz, 2F); ¹³C NMR ((CD₃)₂CO, 126 MHz) δ 153.5, 151.3, 150.3,
146.1 (t, $J_{CF} = 2.1$ Hz), 145.1, 137.3, 123.6, 123.1, 117.6 (tt, $J_{CF} = 270.0, 28.6$ Hz), 109.1 (tt, $J_{CF} = 249.0, 40.7$ Hz); HRMS (ES$^+$) m/z calc’d. for C$_{12}$H$_8$F$_4$N$_5$O$_3$ (M+H)$^+$ 346.0558, found 346.0562.

6-((4-(Chlorodifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12n). Synthesized by procedure 2A to yield 2.12n in 65% as a pale yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.08 (br s, 1H), 9.69 (s, 1H), 8.29 – 8.25 (m, 2H), 7.46 – 7.43 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -26.23 (s, 2F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.5, 151.4, 147.5, 145.1, 137.9, 126.3 (t, $J_{CF} = 286.8$ Hz), 123.6, 122.9; HRMS (ES$^+$) m/z calc’d. for C$_{11}$H$_7$ClF$_2$N$_5$O$_3$ (M+H)$^+$ 330.0200, found 330.0202.

6-((2,2-Difluorobenzo[d][1,3]dioxol-5-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12o). Synthesized by procedure 2A to yield 2.12o in 59% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.08 (br s, 1H), 9.71 (s, 1H), 8.23 (d, $J = 2.2$ Hz, 1H), 7.91 (dd, $J = 8.8$, 2.2 Hz, 1H), 7.38 (d, $J = 8.8$ Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -51.12 (s, 2F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.5, 151.4, 150.2, 145.1, 144.2, 141.1, 135.3, 132.7 (t, $J_{CF} = 252.9$ Hz), 118.1, 110.7, 104.8; HRMS (ES$^+$) m/z calc’d. for C$_{11}$H$_6$F$_2$N$_5$O$_4$ (M+H)$^+$ 310.0382, found 310.0386.

6-((4-(1,1,1,3,3,3-Hexafluoro-2-hydroxypropan-2-yl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12p). In a screw-cap vial, a mixture of 2.11 (0.200 g, 1.05 mmol), 2-(4-aminophenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol (0.125 g, 0.482 mmol), and NaHCO$_3$ (0.100 g, 1.19 mmol) in acetone/H$_2$O (9:1, 4 mL) was stirred at room temperature for 18 h. The mixture was diluted with an aqueous solution of KOH (0.320 g in 4 mL) and stirring was continued for 2 h. The mixture was acidified with 1 M HCl and extracted with EtOAc. The organic layer was washed with brine, dried (Na$_2$SO$_4$), and concentrated to an orange residue. The residue was purified by chromatography on SiO$_2$ (gradient: 0-3% MeOH/CH$_2$Cl$_2$) to yield a sticky yellow solid (0.196 g). The solid was dissolved in a minimal amount of acetone and then precipitated by the addition of
hexanes. The precipitate was filtered, rinsed with hexanes, and collected to yield 2.12p (0.155 g, 81%) as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.08 (br s, 1H), 9.69 (s, 1H), 8.30 – 8.28 (m, 2H), 7.87 (d, $J = 8.7$ Hz, 2H), 7.52 (br s, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -75.64 (s, 6F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.5, 151.5, 150.3, 145.1, 140.4, 128.5, 127.7, 124.1 (q, $J_{CF} = 288.1$ Hz), 122.0, 78.1 (p, $J_{CF} = 29.7$ Hz); HRMS (ES$^+$) m/z calc’d for C$_{13}$H$_8$F$_6$N$_5$O$_3$ (M+H)$^+$ 396.0526, found 396.0530.

6-((2,3-Difluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12q). Synthesized by procedure 2B to yield 2.12q in 29% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.09 (s, 1H), 9.35 (s, 1H), 8.24 – 8.17 (m, 1H), 7.38 – 7.30 (m, 1H), 7.29 – 7.20 (m, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -139.53 – -139.84 (m, 1F), -152.07 – -152.34 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 153.4, 151.8, 151.3 (dd, $J_{CF} = 245.6, 11.0$ Hz), 150.2, 145.3, 143.4 (dd, $J_{CF} = 247.2, 14.6$ Hz), 128.2 (dd, $J_{CF} = 7.8, 1.9$ Hz), 125.4 (dd, $J_{CF} = 8.0, 5.0$ Hz), 120.1 (d, $J_{CF} = 3.5$ Hz), 114.7 (d, $J_{CF} = 16.9$ Hz); HRMS (ESI$^+$) m/z calc’d. for C$_{10}$H$_6$F$_2$N$_5$O$_2$ (M+H)$^+$ 266.0484, found 266.0479.

6-((2,6-Difluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12r). Synthesized by procedure 2B to yield 2.12r in 23% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.67 (s, 1H), 9.41 (s, 1H), 7.54 – 7.45 (m, 1H), 7.22 – 7.15 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -117.87 – -117.95 (m, 2F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 159.4 (dd, $J_{CF} = 250.9, 4.7$ Hz), 153.4, 153.0, 150.3, 145.4, 130.2 (t, $J_{CF} = 9.9$ Hz), 114.7 (t, $J_{CF} = 16.6$ Hz), 112.9 – 112.5 (m); HRMS (ESI$^+$) m/z calc’d. for C$_{10}$H$_6$F$_2$N$_5$O$_2$ (M+H)$^+$ 266.0484, found 266.0480.

6-((3,5-Difluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12s). Synthesized by procedure 2B to yield 2.12s in 19% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.11 (s, 1H), 9.76 (s, 1H), 7.97 – 7.88 (m, 2H), 6.87 (tt, $J = 9.1, 2.3$ Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376
MHz) δ -110.23 – -110.33 (m, 2F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 101 MHz) δ 163.84 (dd, J\(\text{CF} = 244.1, 14.8\) Hz), 153.2, 151.5, 149.9, 145.0, 141.1 (t, J\(\text{CF} = 13.9\) Hz), 105.0 (dd, J\(\text{CF} = 21.1, 9.1\) Hz), 100.6 (t, J\(\text{CF} = 26.2\) Hz); HRMS (ESI\(^+\)) \(m/z\) calc’d for C\(_{10}\)H\(_6\)F\(_2\)N\(_5\)O\(_2\) (M+H\(^+\)) 266.0484, found 266.0488.

6-((2-Fluoro-5-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12t). Synthesized by procedure 2B to yield 2.12t in 26% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) δ 11.99 (s, 1H), 9.40 (s, 1H), 8.90 (dd, \(J = 7.4, 1.9\) Hz, 1H), 7.72 – 7.66 (m, 1H), 7.63 – 7.56 (m, 1H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) δ -62.66 (s, 3F), -122.44 – -122.53 (m, 1F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 126 MHz) δ 157.0 (d, J\(\text{CF} = 252.7\) Hz), 153.5, 151.9, 150.1, 145.4, 127.6 (d, J\(\text{CF} = 4.0\) Hz), 127.4 (d, J\(\text{CF} = 11.6\) Hz), 124.9 (q, J\(\text{CF} = 271.4\) Hz), 124.5 (dd, J\(\text{CF} = 9.5, 4.3\) Hz), 121.5 – 121.4, (m), 117.5 (d, J\(\text{CF} = 21.2\) Hz); HRMS (ESI\(^+\)) \(m/z\) calc’d for C\(_{11}\)H\(_6\)F\(_4\)N\(_5\)O\(_2\) (M+H\(^+\)) 316.0452, found 316.0476.

6-((2-Fluoro-4-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12u). Synthesized by procedure 2B to yield 2.12u in 14% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) δ 11.92 (s, 1H), 9.37 (s, 1H), 8.81 (t, \(J = 8.3\) Hz, 1H), 7.79 – 7.67 (m, 2H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) δ -62.82 (s, 3F), -127.05 (t, \(J = 10.5\) Hz, 1F); \(^{13}\)C NMR (126 MHz, Acetone-\(d_6\)) δ 154.2 (d, \(J = 248.5\) Hz), 153.4, 151.6, 150.0, 145.2, 130.1 (d, \(J = 11.3\) Hz), 127.7 (dd, \(J = 35.9, 7.6\) Hz), 124.4 (dq, \(J = 271.1, 3.1\) Hz), 124.1, 123.0 (p, \(J = 4.3\) Hz), 113.7 (dq, \(J = 23.1, 4.2\) Hz); HRMS (ESI\(^+\)) \(m/z\) calc’d for C\(_{11}\)H\(_6\)F\(_4\)N\(_5\)O\(_2\) (M+H\(^+\)) 316.0452, found 316.0453.

6-((3-Fluoro-4-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12v). Synthesized by procedure 2B to yield 2.12v in 11% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) δ 11.65 (s, 1H), 9.92 (s, 1H), 8.37 (ddd, \(J = 13.4, 2.0, 0.9\) Hz, 1H), 8.18 – 8.12 (m, 1H), 7.82 (d, \(J = 8.5\) Hz, 1H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) δ -61.27 (d, \(J = 12.3\) Hz, 3F), -114.54 – -114.73 (m, 1F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 101 MHz) δ \(*rotamers* 160.6 (dq, J\(\text{CF} = 251.6, 2.5\) Hz),
153.3*, 153.2, 151.8*, 151.7, 149.9, 145.2, 144.4 – 144.0 (m), 128.8 – 128.5 (m), 123.8 (dd, $J_{CF} = 270.0, 2.3$ Hz), 117.6 (d, $J_{CF} = 3.5$ Hz)*, 117.5 (d, $J_{CF} = 3.6$ Hz), 113.9 (dd, $J_{CF} = 33.0, 12.8$ Hz), 109.8 (d, $J_{CF} = 26.3$ Hz)*, 109.7 (d, $J_{CF} = 26.4$ Hz); HRMS (ESI) $m/z$ calc’d. for C$_{11}$H$_{4}$_{4}F$_{4}$N$_{5}$O$_{2}$ (M-H)$^-$ 314.0306, found 314.0352.

6-((2-Fluoro-3-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12w).

Synthesized by procedure 2B to yield 2.12w in 21% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.21 (s, 1H), 9.41 (s, 1H), 8.75 – 8.65 (m, 1H), 7.69 – 7.51 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -61.75 (d, $J = 13.0$ Hz, 3F), -129.74 – -129.93 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ *rotamers 152.4, 152.3*, 151.5 (dd, $J_{CF} = 256.3, 2.4$ Hz), 151.0, 150.9*, 149.1, 149.2*, 144.3, 128.2 (d, $J_{CF} = 18.9$ Hz), 126.8, 125.4 (q, $J_{CF} = 271.6$ Hz), 124.9 (dd, $J_{CF} = 5.1, 2.1$ Hz), 123.0 (dd, $J_{CF} = 4.7, 1.2$ Hz), 117.9 (dd, $J_{CF} = 33.1, 10.8$ Hz); HRMS (ESI-) $m/z$ calc’d. for C$_{11}$H$_{4}$_{4}F$_{4}$N$_{5}$O$_{2}$ (M-H)$^-$ 314.0306, found 314.0352.

6-((3,5-Bis(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12x).

Synthesized by procedure 2B to yield 2.12x in 6% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.20 (s, 1H), 10.07 (s, 1H), 8.89 (d, $J = 1.8$ Hz, 2H), 7.87 (s, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -63.57 (s, 6F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.2, 151.9, 149.9, 145.2, 140.6, 132.7 (q, $J_{CF} = 33.3$ Hz), 124.4 (q, $J_{CF} = 272.0$ Hz), 122.1 (q, $J_{CF} = 4.1$ Hz), 118.6 – 118.5 (m); HRMS (ESI-) $m/z$ Calc’d. for C$_{10}$H$_{4}$BrFN$_{5}$O$_{2}$ (M-H)$^-$ 364.0274, found 364.0261.

6-((2-Fluoro-4-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12y). Synthesized by procedure 2B to yield 2.12y in 23% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.53 (s, 1H), 9.30 (s, 1H), 8.56 (t, $J = 8.9$ Hz, 1H), 7.61 – 7.28 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -59.02 (s, 3F), -123.51 – -123.59 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 155.1 (d, $J_{CF} = 249.9$ Hz), 153.4, 151.7, 150.1, 146.6 (dd, $J_{CF} = 10.7, 2.3$ Hz), 145.3,
125.7 (d, $J_{CF} = 10.7$ Hz), 125.5 (d, $J_{CF} = 2.3$ Hz), 121.3 (q, $J_{CF} = 256.6$ Hz), 118.3 (dd, $J_{CF} = 4.2$, 1.2 Hz), 110.4 (dd, $J_{CF} = 23.6$, 1.3 Hz); HRMS (ESI') $m/z$ calc’d for C$_{11}$H$_4$F$_4$N$_5$O$_3$ (M-H)$^-$ 330.0255, found 330.0257.

6-((3-Fluoro-4-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12z). Synthesized by procedure 2A to yield 2.12z in 51% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) $\delta$ 12.10 (br s, 1H), 9.82 (br s, 1H), 8.40 – 8.36 (m, 1H), 8.08 – 8.04 (m, 1H), 7.62 – 7.58 (m, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -59.87 – -59.88 (m, 3F), -128.86 – -128.96 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) $\delta$ 154.9 (d, $J_{CF} = 248.7$ Hz), 153.3, 151.5, 150.1, 145.1, 139.2 (d, $J_{CF} = 10.0$ Hz), 133.1 (d, $J_{CF} = 12.5$ Hz), 125.2, 121.5 (q, $J_{CF} = 257.2$ Hz), 118.6 (d, $J_{CF} = 3.7$ Hz), 110.9 (d, $J_{CF} = 24.4$ Hz); HRMS (ESI') $m/z$ calc’d for C$_{11}$H$_4$F$_4$N$_5$O$_3$ (M-H)$^-$ 330.0256, found 330.0289.

6-((3-Chloro-4-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12aa). Synthesized by procedure 2A to yield 2.12aa in 51% as a yellow-orange solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.05 (br s, 1H), 9.78 (s, 1H), 8.53 (d, $J = 2.7$ Hz, 1H), 8.21 (dd, $J = 9.1$, 2.6 Hz, 1H), 7.60 (dq, $J = 9.0$, 1.4 Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -58.87 – -58.88 (m, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) $\delta$ 153.3, 151.5, 150.1, 145.2, 141.9 (q, $J_{CF} = 2.0$ Hz), 138.7, 127.8, 124.3, 123.8, 122.1, 121.5 (q, $J_{CF} = 257.3$ Hz); HRMS (ESI') $m/z$ calc’d for C$_{11}$H$_4$ClF$_3$N$_5$O$_3$ (M-H)$^-$ 345.9960, found 345.9949.

6-((2-Chloro-4-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ab). Synthesized by procedure 2B to yield 2.12ab in 9% as a yellow solid (HPLC condition 2): $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) $\delta$ 13.73 (s, 1H), 9.51 (s, 1H), 8.84 (d, $J = 9.3$ Hz, 1H), 7.66 (d, $J = 3.5$ Hz, 1H), 7.58 – 7.50 (m, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -58.99 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) $\delta$ 153.7, 151.4, 150.1, 146.2 (q, $J_{CF} = 2.4$ Hz), 145.3, 134.3, 126.4, 124.4,
123.6 (q, $J_{CF} = 1.3$ Hz), 121.9 (q, $J_{CF} = 1.1$ Hz), 121.4 (q, $J_{CF} = 256.4$ Hz); HRMS (ESI$^+$) m/z calc’d. for $C_{11}H_6ClF_3N_5O_3$ (M+H)$^+$ 348.0105, found 348.0089.

6-((2-Methoxy-4-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ac). Synthesized by procedure 2A to yield 2.12ac in 68% as an orange solid: $^1H$ NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.20 (br s, 1H), 9.46 (s, 1H), 8.86 (d, $J = 8.9$ Hz, 1H), 7.15 (d, $J = 2.6$ Hz, 1H), 7.09 (dt, $J = 9.0, 2.6$ Hz, 1H), 4.10 (s, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -58.70 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.8, 151.1, 150.6, 150.3, 146.5 (q, $J_{CF} = 2.1$ Hz), 145.1, 126.7, 121.5 (q, $J_{CF} = 255.6$ Hz), 121.3, 113.7, 105.7, 57.3; HRMS (ESI$^-$) m/z calc’d. for $C_{11}H_7F_3N_5O_3$ (M-H)$^-$ 342.0456, found 342.0448.

6-((3-Nitro-4-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ad). Synthesized by procedure 2B to yield 2.12ad in 2% as a yellow solid: $^1H$ NMR ((CD$_3$)$_2$CO, 400 MHz) δ 10.06 (s, 1H), 9.05 (d, $J = 2.7$ Hz, 1H), 8.61 (dd, $J = 9.1, 2.7$ Hz, 1H), 7.80 (dq, $J = 9.1, 1.3$ Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -58.769 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 153.4, 151.8, 149.9, 145.4, 143.7, 138.7, 137.3, 127.7, 125.3 (q, $J_{CF} = 1.2$ Hz), 121.3 (q, $J_{CF} = 258.5$ Hz), 118.9; HRMS (ESI$^+$) m/z calc’d. for $C_{11}H_7F_3N_6O_5$ (M-H)$^+$ 357.0200, found 357.0198.

6-((2-Fluorophenyl)(methyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ae). Synthesized by procedure 2A to yield 2.12ae in 42% as a yellow solid: $^1H$ NMR ((CD$_3$)$_2$CO, 500 MHz) δ 11.63 (br s, 1H), 7.47 - 7.42 (m, 1H), 7.40 - 7.34 (m, 1H), 7.26 - 7.20 (m, 2H), 3.54 (s, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -125.48 - -125.68 (m, 1 F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 158.0 (d, $J_{CF} = 245.9$ Hz), 154.6, 152.3, 150.4, 145.7, 134.6 (d, $J_{CF} = 12.8$ Hz), 129.7 (d, $J_{CF} = 7.8$ Hz), 128.7, 125.6 (d, $J_{CF} = 3.9$ Hz), 116.7 (d, $J_{CF} = 20.2$ Hz), 42.6; HRMS (ESI$^+$) m/z calc’d. for $C_{11}H_7F_3N_2O_2$ (M-H)$^-$ 260.0589, found 260.0593.
6-((3-Methoxyphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12af). Synthesized by procedure 2B to yield 2.12af in 36% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$SO, 400 MHz) $\delta$ 13.27 (s, 1H), 10.16 (s, 1H), 7.74 – 7.66 (m, 2H), 7.32 (t, $J$ = 8.2 Hz, 1H), 6.77 (dd, $J$ = 8.0, 2.0 Hz, 1H), 3.77 (s, 3H); $^{13}$C NMR ((CD$_3$)$_2$SO, 126 MHz) $\delta$ 159.34, 153.1, 150.7, 149.6, 144.5, 138.9, 129.5, 113.9, 110.0, 107.7, 55.1; HRMS (ESI$^+$) m/z calc’d. for C$_{11}$H$_{10}$N$_5$O$_3$ (M+H)$^+$ 260.0778, found 260.0793.

6-((3-Butoxyphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ag). In a vial, 1-iodobutane (0.20 mL, 1.8 mmol) was added to a stirring mixture of N-(3-hydroxyphenyl)acetamide (0.150 g, 0.992 mmol) and K$_2$CO$_3$ (0.200 g, 1.45 mmol) in acetone (1.5 mL). The resulting mixture was stirred for 2 d. Additional 1-iodobutane (0.20 mL, 1.8 mmol) was added and the mixture was heated to 40 °C for 9 h. The mixture was diluted with EtOAc, washed with brine, dried (Na$_2$SO$_4$), and concentrated to a clear oil (0.177 g). The oil was heated in a mixture of 6M aq HCl (5 mL) and dioxane (3 mL) at 90 °C for 6 h. The mixture was allowed to cool to rt, basified with 1M NaOH, and extracted with EtOAc (2x). The combined organic layers were washed with brine, dried (Na$_2$SO$_4$), and concentrated to yield 3-butoxyaniline as a crude orange oil (0.123 g).

Product 2.12ag was synthesized using the crude aniline by procedure 2A in 37% as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) $\delta$ 12.04 (br s, 1H), 9.42 (s, 1H), 7.83 (t, $J$ = 2.2 Hz, 1H), 7.68 (ddd, $J$ = 8.2, 2.1, 0.9 Hz, 1H), 7.33 (t, $J$ = 8.2 Hz, 1 H), 6.79 (ddd, $J$ = 8.2, 2.5, 0.9 Hz, 1H), 4.04 (t, $J$ = 6.5 Hz, 2H), 1.82 – 1.75 (m, 2H), 1.56 – 1.47 (m, 2H), 0.98 (t, $J$ = 7.4 Hz, 3H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) $\delta$ 160.5, 153.6, 151.0, 150.4, 145.0, 139.7, 130.5, 114.1, 111.9, 108.4, 68.4, 32.0, 19.9, 14.1; HRMS (ES$^+$) m/z calc’d. for C$_{14}$H$_{16}$N$_5$O$_3$ (M+H)$^+$ 302.1248, found 302.1251.
6-((4-Butoxyphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ah). Synthesized by procedure 2B to yield 2.12ah in 75% as tan solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 11.97 (s, 1H), 9.40 (s, 1H), 8.16 – 7.87 (m, 2H), 7.08 – 6.89 (m, 2H), 4.02 (t, \(J = 6.5\) Hz, 2H), 1.87 – 1.69 (m, 2H), 1.63 – 1.42 (m, 2H), 0.97 (t, \(J = 7.4\) Hz, 3H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 101 MHz) \(\delta\) 156.6, 152.8, 149.7, 149.6, 144.1, 130.6, 122.7, 114.4, 67.6, 31.2, 18.9, 13.2; HRMS (ESI\(^+\)) \(m/\text{z}\) Calc’d. for C\(_{14}\)H\(_{16}\)N\(_5\)O\(_3\) (M+H)\(^+\) 302.1247, found 302.1253.

6-((4-(2,2,2-Trifluoroethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ai). In a round-bottom flask, a mixture of 2.12ai-int and iron (325 mesh, 0.200 g, 3.58 mmol) in AcOH/MeOH (1:1, 4 mL) was stirred at 50 °C for 2 h. The mixture was filtered through Celite, rinsing with EtOAc, and concentrated to a dark oil. The oil was quenched with sat. aq. NaHCO\(_3\), diluted with brine, and extracted with EtOAc. The organic layer was dried (Na\(_2\)SO\(_4\)) and concentrated to yield 4-(2,2,2-trifluoroethoxy)aniline as a crude dark oil (0.539 g).

Compound 2.12ai was synthesized using the crude aniline by procedure 2A in 60% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.00 (br s, 1H), 9.50 (s, 1H), 8.11 – 8.08 (m, 2H), 7.17 – 7.15 (m, 2H), 4.71 (q, \(J_{HF} = 8.6\) Hz, 2H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -74.73 (t, \(J = 8.6\) Hz, 3F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 155.6, 153.7, 151.0, 150.5, 145.1, 133.3, 124.9 (q, \(J_{CF} = 289.2\) Hz), 123.8, 116.1, 66.4 (q, \(J_{CF} = 35.1\) Hz); HRMS (ESI\(^-\)) \(m/\text{z}\) calc’d. for C\(_{12}\)H\(_7\)F\(_3\)N\(_5\)O\(_3\) (M-H\(^-\)) 326.0506, found 326.0508.

6-((3-Methyl-4-(2,2,2-trifluoroethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12aj). In a round-bottom flask, a mixture of 2.12aj-int and iron (325 mesh, 0.200 g, 3.58 mmol) in AcOH/MeOH (1:1, 4 mL) was stirred at 50 °C for 2 h. The mixture was filtered through Celite, rinsing with EtOAc, and concentrated to a dark oil. The oil was quenched with sat. aq. NaHCO\(_3\),
diluted with brine, and extracted with EtOAc. The organic layer was dried (Na$_2$SO$_4$) and concentrated to yield 3-methyl-4-(2,2,2-trifluoroethoxy)aniline as a crude dark oil (0.143 g).

Product 2.12aj was synthesized using the crude aniline by procedure 2A in 66% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 11.98 (s, 1H), 9.40 (s, 1H), 8.00 (dd, $J$ = 8.9, 2.7 Hz, 1H), 7.88 (d, $J$ = 2.7 Hz, 1H), 7.12 (d, $J$ = 8.9 Hz, 1H), 4.70 (q, $J_{HF}$ = 8.5 Hz, 2H), 2.28 (s, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -74.92 (t, $J$ = 8.9 Hz, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.8, 153.7, 150.9, 150.5, 145.1, 133.0, 128.3, 124.99 (q, $J_{CF}$ = 277.3 Hz), 124.98, 121.0, 113.4, 66.7 (q, $J_{CF}$ = 34.8 Hz), 16.2; HRMS (ESI) m/z calc’d. for C$_{13}$H$_9$F$_3$N$_5$O$_3$ (M-H)$^-$ 340.0663, found 340.0667.

6-((3-Fluoro-4-(2,2,2-trifluoroethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ak). Synthesized by procedure 2B using 2.12ak-int to yield 2.12ak in 53% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 9.62 (s, 1H), 8.18 (dd, $J$ = 13.5, 3.0 Hz, 1H), 7.94 – 7.86 (m, 1H), 7.37 (t, $J_{HF}$ = 9.6 Hz , 1H), 4.79 (q, $J_{HF}$ = 8.9 Hz , 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -74.94 (t, $J$ = 8.9 Hz, 3F), -132.89 – -133.04 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ *rotamers 153.5, 153.4*, 152.9 (d, $J_{CF}$ = 244.5 Hz), 151.2, 151.1*, 150.3, 145.1, 143.2 (dd, $J_{CF}$ = 11.1, 1.3 Hz), 134.2 (d, $J_{CF}$ = 9.6 Hz), 124.7 (q, $J_{CF}$ = 276.7 Hz), 118.4 (d, $J_{CF}$ = 3.8 Hz), 118.3 (d, $J_{CF}$ = 3.8 Hz)*, 117.7 (d, $J_{CF}$ = 2.7 Hz), 110.9 (d, $J_{CF}$ = 23.7 Hz), 110.8 (d, $J_{CF}$ = 23.8 Hz)*, 67.7 (q, $J_{CF}$ = 35.6 Hz). HRMS (ESI$^+$) m/z calc’d. for C$_{12}$H$_9$F$_3$N$_5$O$_3$ (M+H)$^+$ 346.0557, found 346.0574.

6-((4-(2,2,2-Trifluoroethoxy)-3-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12al). In a round-bottom flask, a mixture of 2.12al-int (0.200 g) and iron (325 mesh, 0.200 g, 3.58 mmol) in AcOH/MeOH (1:1, 4 mL) was stirred at 50 °C for 2 h. The mixture was filtered through Celite, rinsing with EtOAc, and concentrated to a dark oil. The oil was quenched with sat. aq. NaHCO$_3$, diluted with brine, and extracted with EtOAc. The organic layer
was dried (Na$_2$SO$_4$) and concentrated to yield 4-(2,2,2-trifluoroethoxy)-3-(trifluoromethyl)aniline as a clear oil (0.152 g): $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 7.09 (d, $J = 8.8$ Hz, 1H), 6.97 – 6.96 (m, 1H), 6.92 – 6.89 (m, 1H), 4.83 (br s, 2H), 4.60 (q, $J_{HF} = 8.6$ Hz, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -62.38 (s, 3F), -75.03 (t, $J = 8.6$ Hz, 3F).

Product 2.12a was synthesized using the crude aniline by procedure 2A in 46% as an orange solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.03 (br s, 1H), 9.73 (s, 1H), 8.48 (d, $J = 2.8$ Hz, 1H), 8.44 (dd, $J = 9.0$, 2.8 Hz, 1H), 7.48 (d, $J = 8.9$ Hz, 1H), 4.88 (q, $J_{HF} = 8.4$ Hz, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -62.78 (m, 3F), -74.84 (t, $J = 8.5$ Hz, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.5, 152.8, 151.4, 150.3, 145.2, 133.2, 127.6, 124.6 (q, $J_{CF} = 276.8$ Hz), 124.2 (q, $J_{CF} = 271.5$ Hz), 121.4 (q, $J_{CF} = 5.6$ Hz), 119.8 (q, $J_{CF} = 31.4$ Hz), 115.7, 67.0 (q, $J_{CF} = 35.7$ Hz); HRMS (ESI) m/z calc’d. for C$_{13}$H$_6$F$_6$N$_5$O$_3$ (M-H) - 394.0380, found 394.0384.

6-((3-Fluoro-4-(4,4,4-trifluorobutoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12am) In a sealed microwave vial, a mixture of 1,2-difluoro-4-nitrobenzene (0.350 g, 2.20 mmol), 4,4,4-trifluorobutan-1-ol (0.35 mL, 3.3 mmol), and K$_2$CO$_3$ (0.700 g, 5.07 mmol) in DMF (2 mL) was stirred at 80 °C for 22 h. The mixture was allowed to cool to rt, diluted with water and brine, and extracted with EtOAc (2x). The combined organic layers were dried (Na$_2$SO$_4$) and concentrated to a crude yellow liquid (0.657 g). In a 6-dram vial, a mixture of the liquid (0.657 g) and iron (325 mesh, 0.650 g, 11.6 mmol) in AcOH/MeOH (1:1, 5 mL) was stirred at 50 °C for 2 h under an atmosphere of N$_2$. The mixture was filtered through Celite (EtOAc), quenched with sat. aq. NaHCO$_3$, and extracted with EtOAc (2x). The combined organic layers were dried (Na$_2$SO$_4$) and concentrated. The residue was purified by chromatography on SiO$_2$ (gradient: 20-30% EtOAc/hexanes) to yield 3-fluoro-4-(4,4,4-trifluorobutoxy)aniline (0.421 g) as a crude orange liquid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 6.86 (dd, $J = 9.5$, 8.7 Hz, 1H), 6.48 (dd, $J = 13.4$, 2.6 Hz,
1H), 6.39 (dd, J = 8.7, 2.7, 1.3 Hz, 1H), 4.57 (s, 2H), 4.01 (t, J = 6.1 Hz, 2H), 2.49 – 2.36 (m, 2H), 2.00 – 1.93 (m, 2H); 19F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -67.05 (t, J = 11.2 Hz, 3F), -134.85 – -134.91 (m, 1F).

Product 2.12am was synthesized using the crude aniline by procedure 2A in 60% as a yellow solid:

$^1$H NMR ((CD$_3$)$_2$SO, 500 MHz) δ 13.28 (br s, 1H), 10.34 (s, 1H), 8.01 (dd, J = 13.7, 2.6 Hz, 1H), 7.85 (ddd, J = 9.0, 2.5, 1.3 Hz, 1H), 7.23 (t, J = 9.3 Hz, 1H), 4.13 (t, J = 6.2 Hz, 2H), 2.51 – 2.38 (m, 2H), 1.99 – 1.93 (m, 2H); 19F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -67.01 (t, J = 11.3 Hz, 3F), -133.97 – -134.035 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$SO, 125 MHz) δ 152.9, 150.9 (d, J$_{CF}$ = 242.1 Hz), 150.5, 149.6, 144.5, 142.9 (d, J$_{CF}$ = 10.8 Hz), 131.5 (d, J$_{CF}$ = 9.6 Hz), 127.6 (q, J$_{CF}$ = 276.1 Hz), 117.9 (d, J$_{CF}$ = 3.4 Hz), 115.2 (d, J$_{CF}$ = 2.5 Hz), 109.9 (d, J$_{CF}$ = 23.1 Hz), 67.4, 29.4 (q, J$_{CF}$ = 28.0 Hz), 21.6 (q, J$_{CF}$ = 3.1 Hz); HRMS (ES') m/z calc'd. for C$_{14}$H$_{10}$F$_4$N$_5$O$_3$ (M-H) - 372.0725, found 372.0726.

6-((4-(4,4,4-Trifluorobutoxy)-3-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12an). In a sealed microwave vial, a mixture of 1-fluoro-4-nitro-2-(trifluoromethyl)benzene (0.400 g, 1.91 mmol), 4,4,4-trifluorobutan-1-ol (0.35 mL, 3.3 mmol), and K$_2$CO$_3$ (0.700 g, 5.07 mmol) in DMF (2 mL) was stirred at 80 °C for 22 h. The mixture was allowed to cool to rt, diluted with water and brine, and extracted with EtOAc (2x). The combined organic layers were dried (Na$_2$SO$_4$) and concentrated to a crude orange liquid (0.913 g). In a 6-dram vial, a mixture of the liquid (0.913 g) and iron (325 mesh, 0.650 g, 11.6 mmol) in AcOH/MeOH (1:1, 5 mL) was stirred at 50 °C for 2 h under an atmosphere of N$_2$. The mixture was filtered through Celite (EtOAc), quenched with sat. aq. NaHCO$_3$, and extracted with EtOAc (2x). The combined organic layers were dried (Na$_2$SO$_4$) and concentrated. The residue was purified by chromatography on SiO$_2$ (gradient: 20-30% EtOAc/hexanes) to yield 4-(4,4,4-
trifluorobutoxy)-3-(trifluoromethyl)aniline (0.452 g) as a crude orange liquid that solidified to an offwhite solid: \(^1\)H NMR \(((\text{CD}_3)_2\text{CO}, 400 \text{ MHz})\) \(\delta\) 6.99 – 6.96 (m, 1H), 6.95 – 6.94 (m, 1H), 6.90 – 6.86 (m, 1H), 4.65 (br s, 2H), 4.10 – 4.07 (m, 2H), 2.49 – 2.36 (m, 2H), 2.04 – 1.98 (m, 2H); \(^19\)F NMR \(((\text{CD}_3)_2\text{CO}, 376 \text{ MHz})\) \(\delta\) -62.81 (d, \(J = 4.0 \text{ Hz}\), 3F), -67.13 (dt, \(J = 11.0, 3.5 \text{ Hz}\), 3F).

Product 2.12an was synthesized using the crude aniline by procedure 2A in 53% as a yellow solid: \(^1\)H NMR \(((\text{CD}_3)_2\text{SO}, 500 \text{ MHz})\) \(\delta\) 13.28 (br s, 1H), 10.45 (s, 1H), 8.37 (d, \(J = 2.6 \text{ Hz}\), 1H), 8.28 (dd, \(J = 9.1, 2.7 \text{ Hz}\), 1H), 7.34 (d, \(J = 9.1 \text{ Hz}\), 1H), 4.20 (t, \(J = 6.0 \text{ Hz}\), 2H), 2.47 – 2.37 (m, 2H), 2.00 – 1.94 (m, 2H); \(^19\)F NMR \(((\text{CD}_3)_2\text{CO}, 376 \text{ MHz})\) \(\delta\) -62.81 (s, 3F), -67.09 (t, \(J_{HF} = 11.5 \text{ Hz}\), 3F); \(^{13}\)C NMR \(((\text{CD}_3)_2\text{SO}, 125 \text{ MHz})\) \(\delta\) 153.0, 152.8, 150.8, 149.6, 144.6, 130.8, 127.6 (q, \(J_{CF} = 276.0 \text{ Hz}\), 127.4, 123.5 (q, \(J_{CF} = 272.1 \text{ Hz}\), 120.5 (q, \(J_{CF} = 5.9 \text{ Hz}\), 116.8 (q, \(J_{CF} = 30.4 \text{ Hz}\), 114.0, 66.8, 29.3 (q, \(J_{CF} = 28.3 \text{ Hz}\), 21.6 (q, \(J_{CF} = 3.2 \text{ Hz}\); HRMS (ES\textsuperscript{+}) \(m/z\) calc’d. for C\(_{15}\)H\(_{10}\)F\(_6\)N\(_5\)O\(_3\) (M – H)\(^–\) 422.0693, found 422.0696.

6-((4-(Cyclopropylmethoxy)-3-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol) (2.12ao). In a sealed vial, a mixture of 1-fluoro-4-nitro-2-(trifluoromethyl)benzene (0.250 g, 1.20 mmol), potassium carbonate (0.400 g, 2.89 mmol), and cyclopropylmethanol (0.25 mL, 3.1 mmol) in DMF (2 mL) was stirred at 80 °C for 22 h. The mixture was allowed to cool to rt, diluted with water and brine, and extracted with EtOAc (2x). The combined organic layers were washed with brine (2x), dried (Na\(_2\)SO\(_4\)), and concentrated to a crude oil. A mixture of the oil and iron (325 mesh, 0.300 g, 5.37 mmol) in AcOH/MeOH (1:1, 3 mL) was stirred at 50 °C for 2 h under an atmosphere of N\(_2\). The mixture was quenched with sat. aq. NaHCO\(_3\) and extracted with EtOAc (2x). The combined organic layers were washed with sat. aq. NaHCO\(_3\) (2x) and then brine, dried (Na\(_2\)SO\(_4\)), and concentrated to yield 4-(cyclopropylmethoxy)-3-(trifluoromethyl)aniline as a yellow oil (0.240 g). The oil was used crude
in the next reaction. \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 6.95 – 6.92 (m, 2H), 6.87 – 6.83 (m, 1H), 4.59 (br s, 2H), 3.84 (d, \(J = 6.5\) Hz, 2H), 1.25 – 1.15 (m, 1H), 0.57 – 0.52 (m, 2H), 0.36 – 0.31 (m, 2H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -62.20 (s, 3F).

Product **2.12ao** was synthesized using the crude aniline by procedure **2A** in 40% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.02 (br s, 1H), 9.65 (s, 1H), 8.40 (d, \(J = 2.8\) Hz, 1H), 8.34 (dd, \(J = 9.0, 2.8\) Hz, 1H), 7.31 (d, \(J = 9.0\) Hz, 1H), 4.07 (d, \(J = 6.6\) Hz, 2H), 1.34 – 1.28 (m, 1H), 0.63 – 0.60 (m, 2H), 0.43 – 0.40 (m, 2H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -62.71 (s, 3F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 155.0 (q, \(J_{CF} = 1.9\) Hz), 153.5, 151.3, 150.4, 145.1, 131.4, 127.6, 124.6 (q, \(J_{CF} = 271.9\) Hz), 121.3 (q, \(J_{CF} = 5.5\) Hz), 119.2 (q, \(J_{CF} = 30.7\) Hz), 115.1, 74.1, 10.7, 3.3; HRMS (ESI\(^+\)) m/z calc’d. for C\(_{15}\)H\(_{13}\)F\(_3\)N\(_5\)O\(_3\) (M+H)\(^+\) 368.0965, found 368.0966.

6-(Benzo[d][1,3]dioxol-5-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (**2.12ap**). Synthesized by procedure **2A** to yield **2.12ap** in 60% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.00 (br s, 1H), 9.44 (s, 1H), 7.77 (d, \(J = 2.3\) Hz, 1H), 7.56 (dd, \(J = 8.5, 2.2\) Hz, 1H), 6.91 (d, \(J = 8.5\) Hz, 1H), 6.05 (s, 2H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 153.6, 150.8, 150.5, 148.7, 145.8, 145.0, 145.8, 145.0, 132.9, 115.6, 108.8, 103.9, 102.5; HRMS (ESI\(^-\)) m/z calc’d. for C\(_{11}\)H\(_{6}\)N\(_5\)O\(_4\) (M-H)\(^-\) 272.0425, found 272.0430.

6-((4-(Benzyloxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (**2.12aq**). Synthesized by procedure **2A** at 75 °C to yield **2.12aq** in 30% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 11.99 (br s, 1H), 9.45 (s, 1H), 8.05 – 8.02 (m, 2H), 7.52 – 7.50 (m, 2H), 7.42 – 7.39 (m, 2H), 7.35 – 7.32 (m, 1H), 7.13 – 7.09 (m, 2H), 5.17 (s, 2H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 157.1, 153.7, 150.8, 150.6, 145.1, 138.3, 131.9, 129.3, 128.7, 128.5, 123.7, 115.8, 70.7; HRMS (ESI\(^+\)) m/z calc’d. for C\(_{17}\)H\(_{14}\)N\(_5\)O\(_3\) (M+H)\(^+\) 336.1091, found 336.1088.
6-((3-((4-(Trifluoromethyl)benzyl)oxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-\(b\)]pyrazin-5-ol (2.12ar). Synthesized by procedure 2A using 2.12ar-int to yield 2.12ar in 60% as an off-white solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.06 (br s, 1H), 9.45 (s, 1H), 7.98 (t, \(J = 2.3\) Hz, 1H), 7.76 (app s, 4H), 7.70 (d, \(J = 8.2\) Hz, 1H), 7.36 (d, \(J = 8.3\) Hz, 1H), 6.90 (dd, \(J = 8.3\), 2.4 Hz, 1H), 5.29 (s, 2H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -62.9 (s, 3F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 159.8, 153.5, 151.1, 150.3, 145.0, 142.9, 139.8, 130.6, 130.2 (q, \(J_{CF} = 32.1\) Hz), 128.9, 126.2 (q, \(J_{CF} = 3.9\) Hz), 125.3 (q, \(J_{CF} = 271.3\) Hz), 114.7, 112.3, 108.7, 69.7; HRMS (ES\(^+\)) \(m/z\) calc’d. for C\(_{18}\)H\(_{13}\)F\(_3\)N\(_5\)O\(_3\) (M+H)\(^+\) 404.0965, found 404.0961.

6-((4-(Methylsulfonyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-\(b\)]pyrazin-5-ol (2.12as). Synthesized by procedure 2A to yield 2.12as (94% purity using HPLC condition 1) in 40% as a light yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)SO, 500 MHz) \(\delta\) 13.37 (br s, 1H), 10.60 (s, 1H), 8.31 (d, \(J = 8.8\) Hz, 2H), 7.96 (d, \(J = 8.8\) Hz, 2H), 3.21 (s, 3H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)SO, 125 MHz) \(\delta\) 152.9, 151.3, 149.5, 144.7, 142.3, 136.0, 127.9, 121.7, 43.7; HRMS (ES\(^-\)) \(m/z\) calc’d. for C\(_{11}\)H\(_8\)N\(_5\)O\(_4\)S (M-H)\(^-\) 306.0302, found 306.0307.

6-((3-(Pentafluoro-l6-sulfaneyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-\(b\)]pyrazin-5-ol (2.12at). Synthesized by procedure 2A to yield 2.12at in 37% as a colorless solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 12.05 (br s, 1H), 9.88 (s, 1H), 8.77 – 8.76 (m, 1H), 8.46 – 8.42 (m, 1H), 7.74 – 7.69 (m, 2H); \(^{19}\)F NMR (376 MHz, (CD\(_3\))\(_2\)CO) \(\delta\) 84.0 (p, \(J = 148.0\) Hz, 4F), 62.0 (d, \(J = 148.6\) Hz, 1F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 126 MHz) \(\delta\) 154.6 (p, \(J_{CF} = 17.1\) Hz), 153.4, 151.7, 150.1, 145.2, 139.4, 130.5, 125.6, 122.9 (p, \(J_{CF} = 4.3\) Hz), 119.7 (p, \(J_{CF} = 4.9\) Hz); HRMS (ES\(^+\)) \(m/z\) calc’d. for C\(_{10}\)H\(_7\)F\(_3\)N\(_5\)O\(_2\)S (M+H)\(^+\) 356.0235, found 356.0232.

6-((4-(Pentafluoro-l6-sulfaneyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-\(b\)]pyrazin-5-ol (2.12au). Synthesized by procedure 2A to yield 2.12au in 38% as a colorless cloth-like solid: \(^1\)H NMR
((CD$_3$)$_2$CO, 500 MHz) δ 12.10 (br s, 1H), 9.83 (s, 1H), 8.38 (d, J = 8.9 Hz, 2H), 7.99 – 7.96 (m, 2H); 19F NMR (376 MHz, (CD$_3$)$_2$CO) δ 85.0 (d, J = 147.3 Hz, 4F), 62.9 (d, J = 148.8 Hz, 1F); 13C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.4, 151.6, 150.1 (p, J$_{CF}$ = 17.5 Hz), 145.2, 141.8, 127.8 (p, J$_{CF}$ = 4.8 Hz), 121.9; HRMS (ESI$^-$) m/z calc’d. for C$_{10}$H$_5$F$_5$N$_5$O$_2$S (M-H)$^-$ 354.0090, found 354.0080.

6-((4-((Trifluoromethyl)thio)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12av). Synthesized by procedure 2A to yield 2.12av in 23% as a pale yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.11 (br s, 1H), 9.74 (s, 1H), 8.33 – 8.31 (m, 2H), 7.82 – 7.80 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -44.23 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.4, 151.5, 150.2, 145.1, 141.6, 138.1, 130.8 (q, J$_{CF}$ = 307.1 Hz), 123.0, 119.7 (q, J$_{CF}$ = 2.2 Hz); HRMS (ESI$^-$) m/z calc’d. for C$_{11}$H$_5$F$_3$N$_5$O$_2$S (M-H)$^-$ 328.0122, found 328.0113.

3-((6-Hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)benzonitrile (2.12aw). Synthesized by procedure 2B to yield 2.12aw in 17% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.09 (s, 1H), 9.78 (s, 1H), 8.63 – 8.59 (m, 1H), 8.42 (dd, J = 8.3, 1.1 Hz, 1H), 7.69 (dd, J = 7.9, 0.5 Hz, 1H), 7.62 (dd, J = 7.9, 1.3 Hz, 1H); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 153.4, 151.7, 150.1, 145.2, 139.7, 131.1, 129.1, 126.6, 125.0, 119.1, 113.6; HRMS (ESI$^+$) m/z calc’d. for C$_{11}$H$_7$N$_6$O$_2$ (M+H)$^+$ 255.0625, found 255.0639.

6-(Phenylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ax). Synthesized by procedure 2B to yield 2.12ax in 39% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.04 (s, 1H), 9.50 (s, 1H), 8.12 (dd, J = 9.0, 1.2 Hz, 2H), 7.46 (t, J = 7.8 Hz, 2H), 7.23 (t, J = 7.7 Hz, 1H); $^{13}$C NMR ((CD$_3$)$_2$SO, 126 MHz) δ 153.0, 150.8, 149.7, 144.5, 137.8, 128.6, 124.8, 121.8; HRMS (ESI$^+$) m/z calc’d. for C$_{10}$H$_8$N$_5$O$_2$ (M+H)$^+$ 230.0672, found 230.0671.
6-(Methyl(phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ay). Synthesized by procedure 2A to yield 2.12ay in 93% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 11.50 (br s, 1H), 7.42 – 7.37 (m, 2H), 7.34 – 7.29 (m, 3H), 3.54 (s, 3H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 126 MHz) \(\delta\) 154.6, 152.3, 150.6, 147.4, 145.8, 129.8, 127.7, 126.5, 43.5; HRMS (ESI\(^{-}\)) \(m/z\) calc’d. for C\(_{11}\)H\(_8\)N\(_5\)O\(_2\) (M-H)\(^{-}\) 242.0683, found 242.0694.

6-(Naphthen-2-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12az). Synthesized by procedure 2A to yield 2.12az in 17% as an orange solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.00 (br s, 1H), 9.66 (s, 1H), 8.86 (d, \(J = 2.3\) Hz, 1H), 8.07 (dd, \(J = 8.9, 2.3\) Hz, 1H), 8.09 – 7.90 (m, 3H), 7.54 (t, \(J = 7.5\) Hz, 1H), 7.49 (t, \(J = 7.5\) Hz, 1H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 126 MHz) \(\delta\) 153.7, 151.3, 150.5, 145.2, 136.2, 134.7, 132.0, 129.5, 128.8, 128.5, 127.6, 126.4, 122.0, 119.1; HRMS (ESI\(^{+}\)) \(m/z\) calc’d. for C\(_{14}\)H\(_{10}\)N\(_5\)O\(_2\) (M+H)\(^{+}\) 280.0829, found 280.0835.

6-(Naphthen-1-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ba). Synthesized by procedure 2A to yield 2.12ba in 46% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.10 (br s, 1H), 9.77 (s, 1H), 8.14 (d, \(J = 7.5\) Hz, 1H), 8.02 – 8.00 (m, 1H), 7.90 (d, \(J = 8.2\) Hz, 1H), 7.64 – 7.57 (m, 3H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 153.9, 152.9, 150.7, 145.3, 135.2, 133.5, 129.5, 129.0, 127.4, 127.2, 126.5, 123.0, 122.7; HRMS (ESI\(^{-}\)) \(m/z\) calc’d. for C\(_{14}\)H\(_8\)N\(_5\)O\(_2\) (M-H)\(^{-}\) 278.0683, found 278.0689.

6-(p-Tolylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bb). Synthesized by procedure 2B to yield 2.12bb in 39% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 11.96 (s, 1H), 9.42 (s, 1H), 7.98 (d, \(J = 8.6\) Hz, 2H), 7.26 (d, \(J = 8.4\) Hz, 2H), 2.34 (s, 3H); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 101 MHz) \(\delta\) 153.7, 150.9, 150.5, 145.0, 136.1, 135.5, 130.2, 122.0, 20.9; HRMS (ESI\(^{+}\)) \(m/z\) calc’d. for C\(_{11}\)H\(_{10}\)N\(_5\)O\(_2\) (M+H)\(^{+}\) 244.0829, found 244.0812.
6-((2,6-Dimethylphenyl)amino)\textsubscript{-}[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-\textit{ol} (2.12bc). Synthesized by procedure 2B to yield 2.12bc in 39% as a yellow solid: \textsuperscript{1}H NMR ((CD\textsubscript{3})\textsubscript{2}CO, 400 MHz) δ 11.97 (s, 1H), 9.25 (s, 1H), 7.21 – 7.13 (m, 3H), 2.26 (s, 6H); \textsuperscript{13}C NMR ((CD\textsubscript{3})\textsubscript{2}CO, 101 MHz) δ 153.5, 152.9, 150.9, 145.4, 136.7, 135.2, 128.9, 128.5, 18.4; HRMS (ESI\textsuperscript{+}) \textit{m/z} calc’d. for C\textsubscript{12}H\textsubscript{12}N\textsubscript{5}O\textsubscript{2} (M+H\textsuperscript{+}) 258.0985, found 258.0993.

6-((3,4-Dimethylphenyl)amino)\textsubscript{-}[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-\textit{ol} (2.12bd). Synthesized by procedure 2B to yield 2.12bd in 48% as a yellow solid: \textsuperscript{1}H NMR ((CD\textsubscript{3})\textsubscript{2}CO, 400 MHz) δ 11.93 (s, 1H), 9.32 (s, 1H), 7.86 (dd, \textit{J} = 8.2, 2.4 Hz, 1H), 7.81 (d, \textit{J} = 2.4 Hz, 1H), 7.19 (d, \textit{J} = 8.2 Hz, 1H), 2.28 (s, 3H), 2.25 (s, 3H); \textsuperscript{13}C NMR ((CD\textsubscript{3})\textsubscript{2}CO, 101 MHz) δ 153.7, 150.9, 150.6, 145.1, 137.9, 136.4, 134.4, 130.7, 123.2, 119.6, 20.1, 19.3. HRMS (ESI\textsuperscript{-}) \textit{m/z} calc’d. for C\textsubscript{12}H\textsubscript{10}N\textsubscript{5}O\textsubscript{2} (M-H\textsuperscript{−}) 256.0840, found 256.0847.

6-((3-Ethylphenyl)amino)\textsubscript{-}[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-\textit{ol} (2.12be). Synthesized by procedure 2A to yield 2.12be in 86% as a yellow solid: \textsuperscript{1}H NMR ((CD\textsubscript{3})\textsubscript{2}CO, 500 MHz) δ 12.04 (br s, 1H), 9.42 (br s, 1H), 7.98 (dd, \textit{J} = 8.3, 2.3 Hz, 1H), 7.93 (t, \textit{J} = 2.0 Hz, 1H), 7.36 (t, \textit{J} = 7.9 Hz, 1H), 7.09 (d, \textit{J} = 7.6 Hz, 1H), 2.69 (q, \textit{J} = 7.6 Hz, 2H), 1.25 (t, \textit{J} = 7.6 Hz, 3H); \textsuperscript{13}C NMR ((CD\textsubscript{3})\textsubscript{2}CO, 126 MHz) δ *rotamers 153.64, 153.60*, 151.1, 151.0*, 150.5, 145.9, 145.0, 138.64, 138.55*, 129.7, 125.5, 121.5, 121.4*, 119.5, 119.4*, 29.5, 15.9; HRMS (ESI\textsuperscript{−}) \textit{m/z} calc’d. for C\textsubscript{12}H\textsubscript{10}N\textsubscript{5}O\textsubscript{2} (M-H\textsuperscript{−}) 256.0840, found 256.0856.

6-((4-Ethylphenyl)amino)\textsubscript{-}[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-\textit{ol} (2.12bf). Synthesized by procedure 2B to yield 2.12bf in 56% as a yellow solid. \textsuperscript{1}H NMR ((CD\textsubscript{3})\textsubscript{2}CO, 400 MHz) δ 11.92 (s, 1H), 9.44 (s, 1H), 8.07 – 7.94 (m, 2H), 7.33 – 7.26 (m, 2H), 2.66 (q, \textit{J} = 7.6 Hz, 2H), 1.23 (t, \textit{J} = 7.6 Hz, 3H); \textsuperscript{13}C NMR ((CD\textsubscript{3})\textsubscript{2}CO 101 MHz) δ *rotamers 153.6, 153.6*, 150.9, 150.8*, 150.5,
145.0, 142.0, 141.9*, 136.3, 136.2, 129.0, 122.1, 122.1, 28.9, 16.0; HRMS (ESI⁺) m/z calc’d. for C₁₂H₁₂N₅O₂ (M+H)⁺ 258.0985, found 258.0984.

6-((3-(tert-Butyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bg). Synthesized by procedure 2B to yield 2.12bg in 47% as a yellow solid: ¹H NMR ((CD₃)₂CO, 400 MHz) δ 11.93 (s, 1H), 9.53 – 9.41 (m, 1H), 8.11 – 8.05 (m, 2H), 7.41 – 7.35 (m, 1H), 7.31 – 7.25 (m, 1H), 1.35 (s, 9H); ¹³C NMR ((CD₃)₂CO, 101 MHz) δ 153.6, 152.8, 151.0, 150.5, 145.0, 138.4, 129.4, 122.9, 119.4, 119.2, 35.4, 31.6; HRMS (ESI⁻) m/z calc’d. for C₁₄H₁₄N₅O₂ (M⁻H)⁻ 284.1153, found 284.1152.

6-((4-(tert-Butyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bh). Synthesized by procedure 2B to yield 2.12bh in 64% as a tan solid: ¹H NMR ((CD₃)₂CO, 400 MHz) δ 11.98 (s, 1H), 9.44 (s, 1H), 8.24 – 7.79 (m, 2H), 7.71 – 7.27 (m, 2H), 1.34 (s, 9H); ¹³C NMR ((CD₃)₂CO, 101 MHz) δ 152.7, 150.1, 149.6, 147.9, 144.1, 135.1, 125.6, 120.9, 34.1, 30.7; HRMS (ESI⁺) m/z calc’d. for C₁₄H₁₆N₅O₂ (M+H)⁺ 286.12985, found 286.1289.

6-(Mesitylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bi). Synthesized by procedure 2B to yield 2.12bi in 25% as a colorless solid: ¹H NMR ((CD₃)₂CO, 400 MHz) δ 11.83 (s, 1H), 9.17 (s, 1H), 6.96 (s, 2H), 2.28 (s, 3H), 2.21 (s, 6H); ¹³C NMR ((CD₃)₂CO, 101 MHz) δ 153.6, 153.0, 151.1, 145.5, 138.1, 136.4, 132.7, 129.6, 21.1, 18.4; HRMS (ESI⁻) m/z calc’d. for C₁₃H₁₂N₅O₂ (M⁻H)⁻ 270.0996, found 270.0992.

6-((4-Butylphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bj).Synthesized by procedure 2B to yield 2.12bj in 33% as a yellow solid: ¹H NMR ((CD₃)₂CO, 400 MHz) δ 12.04 (s, 1H), 9.42 (s, 1H), 8.25 – 7.74 (m, 2H), 7.50 – 7.01 (m, 2H), 2.67 – 2.58 (m, 2H), 1.66 – 1.55 (m, 2H), 1.43 – 1.30 (m, 2H), 0.93 (t, J = 7.4 Hz, 3H); ¹³C NMR ((CD₃)₂CO, 101 MHz) δ *rotamers
153.6, 153.5*, 150.8, 150.7*, 144.9, 140.6, 136.2, 136.1*, 129.5, 122.0, 121.9*, 35.6, 34.4, 22.9, 14.2; HRMS (ESI+) m/z calc’d. for C_{14}H_{16}N_{5}O_{2} (M+H)^+ 286.1298, found 286.1307.

6-((4-Pentylphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bk). Synthesized by procedure 2B to yield 2.12bk in 55% as a yellow solid: ^1H NMR ((CD_{3})_2CO, 400 MHz) δ 11.95 (s, 1H), 9.43 (s, 1H), 8.05 – 7.97 (m, 2H), 7.31 – 7.24 (m, 2H), 2.63 (t, J = 7.7 Hz, 2H), 1.69 – 1.58 (m, 2H), 1.42 – 1.28 (m, 4H), 0.89 (t, J = 6.8 Hz, 3H); ^13C NMR ((CD_{3})_2CO, 101 MHz) δ 153.7, 150.9, 150.5, 145.0, 140.7, 136.3, 129.6, 122.1, 35.9, 32.2, 32.0, 23.2, 14.3; HRMS (ESI+) m/z calc’d. for C_{15}H_{18}N_{5}O_{2} (M+H)^+ 300.1455, found 300.1443.

6-((4-Hexylphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bl). Synthesized by procedure 2B to yield 2.12bl in 40% as a yellow solid: ^1H NMR ((CD_{3})_2CO, 400 MHz) δ 11.98 (s, 1H), 9.43 (s, 1H), 8.04 – 7.97 (m, 2H), 7.31 – 7.25 (m, 2H), 2.63 (t, J = 7.5 Hz, 2H), 1.69 – 1.57 (m, 2H), 1.41 – 1.25 (m, 6H), 0.88 (t, J = 6.9 Hz, 3H); ^13C NMR ((CD_{3})_2CO, 101 MHz) δ 153.7, 150.9, 150.5, 145.1, 140.7, 136.3, 129.6, 122.1, 36.0, 32.5, 32.3, 29.7, 23.3, 14.4; HRMS (ESI+) m/z calc’d. for C_{16}H_{20}N_{5}O_{2} (M+H)^+ 314.1611, found 314.1602.

6-((4-Ethyl-2-fluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bm). Synthesized by procedure 2B to yield 2.12bm in 33% as a yellow solid: ^1H NMR ((CD_{3})_2CO, 400 MHz) δ 11.54 (s, 1H), 9.17 (s, 1H), 8.35 (t, J = 8.3 Hz, 1H), 7.23 – 7.13 (m, 2H), 2.69 (q, J = 7.6 Hz, 2H), 1.25 (t, J = 7.6 Hz, 3H); ^19F NMR ((CD_{3})_2CO, 376 MHz) δ rotamers -129.38 – -129.48 (m), -129.63 – -129.71 (m); ^13C NMR ((CD_{3})_2CO, 101 MHz) δ 155.0 (d, J_{CF} = 245.5 Hz), 153.6, 151.4, 150.4, 145.2, 144.4 (d, J_{CF} = 6.9 Hz), 124.8 (d, J_{CF} = 3.4 Hz), 124.1 (d, J_{CF} = 16.1 Hz), 123.8 (d, J_{CF} = 10.7 Hz), 115.5 (d, J_{CF} = 19.0 Hz), 28.9 (d, J_{CF} = 1.6 Hz), 15.7; HRMS (ESI+) m/z calc’d. for C_{12}H_{11}FN_{5}O_{2} (M+H)^+ 276.0891, found 276.0893.
6-((4-Butyl-2-fluorophenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bn). 

Synthesized by procedure 2B to yield 2.12bn in 21% as a yellow solid (HPLC condition 2): 1H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.13 (s, 1H), 9.19 (s, 1H), 8.41 – 8.33 (m, 1H), 7.20 – 7.14 (m, 2H), 2.72 – 2.62 (t, $J = 7.6$ Hz, 2H), 1.69 – 1.58 (m, 2H), 1.44 – 1.31 (m, 2H), 0.94 (t, $J = 7.4$ Hz, 3H); 19F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -129.46 – -129.54 (m); HRMS (ESI$^+$) m/z calc’d. for C$_{14}$H$_{15}$FN$_5$O$_2$ (M+H)$^+$ 304.1204, found 304.1207.

6-((2-Fluoro-4-pentylphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bo). 

Synthesized by procedure 2B with 2.12bo-int2 to yield 2.12bo in 37% as a yellow solid: 1H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.96 (s, 1H), 9.17 (s, 1H), 8.37 (q, $J = 8.1$ Hz, 1H), 7.20 – 7.13 (m, 2H), 2.66 (t, $J = 7.7$ Hz , 2H), 1.72 – 1.60 (m, 2H), 1.40 – 1.30 (m, 4H), 0.89 (t, $J = 7.0$ Hz, 3H); 19F NMR ((CD$_3$)$_2$CO, 376 MHz) δ rotamers -129.56 – -129.65 (m), -129.80 – -129.89 (m); 13C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 154.9 (d, $J_{CF} = 245.6$ Hz), 153.6, 151.3, 150.4, 145.2, 143.0 (d, $J_{CF} = 7.1$ Hz), 125.4 (d, $J_{CF} = 3.2$ Hz), 124.0, 123.8 (d, $J_{CF} = 10.8$ Hz), 115.9 (d, $J_{CF} = 18.9$ Hz), 35.9 (d, $J_{CF} = 1.5$ Hz), 32.1, 31.7, 23.2, 14.3; HRMS (ESI$^+$) m/z calc’d. for C$_{15}$H$_{17}$FN$_5$O$_2$ (M+H)$^+$ 318.1360, found 318.1365.

6-((2-Fluoro-4-hexylphenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bp). 

Synthesized by procedure 2B with 2.12bp-int2 to yield 2.12bp in 50% as a yellow solid: 1H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.91 (s, 1H), 9.16 (s, 1H), 8.35 (t, $J = 8.3$ Hz, 1H), 7.19 – 7.13 (m, 2H), 2.66 (t, $J = 7.6$ Hz , 2H), 1.70 – 1.60 (m, 2H), 1.41 – 1.26 (m, 6H), 0.89 (t, $J = 7.0$ Hz, 3H); 19F NMR ((CD$_3$)$_2$CO, 376 MHz) δ rotamers -129.58 – -129.71 (m), -129.83 – -129.92 (m); 13C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 154.9 (d, $J_{CF} = 245.6$ Hz), 153.6, 151.3, 150.3, 145.1, 142.9 (d, $J_{CF} = 7.0$ Hz), 125.4 (d, $J_{CF} = 3.1$ Hz), 123.9, 123.8 (d, $J_{CF} = 10.7$ Hz), 115.9 (d, $J_{CF} = 18.7$ Hz), 35.9 (d, $J_{CF}$
= 1.4 Hz), 32.4, 31.9, 29.6, 23.3, 14.4; HRMS (ESI+) m/z calc’d. for C_{16}H_{19}FN_{5}O_{2} (M+H)^{+} 332.1517, found 332.1515.

6-((4-Pentyl-2-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bq). Synthesized by procedure 2B with 2.12bq-int2 to yield 2.12bq in 47% as a yellow solid:

$^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.19 (s, 1H), 9.28 (s, 1H), 8.61 (d, $J = 8.5$ Hz, 1H), 7.42 – 7.34 (m, 2H), 2.71 (t, $J = 7.9$ Hz, 2H), 1.72 – 1.62 (m, 2H), 1.40 – 1.30 (m, 4H), 0.89 (t, $J = 6.9$ Hz, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ rotamers -58.42 (d, $J = 1.3$ Hz), -58.44 (d, $J = 1.3$ Hz);

$^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 152.7, 150.2, 149.3, 144.2, 141.5, 139.4, 127.8 (q, $J_{CF} = 1.5$ Hz), 127.4, 122.5, 120.8 (q, $J_{CF} = 259.2$ Hz), 120.8, 34.9, 31.2, 30.8, 22.2, 13.4; HRMS (ESI+) m/z calc’d. for C$_{16}$H$_{17}$F$_3$N$_5$O$_3$ (M+H)$^+$ 384.1278, found 384.1296.

6-((4-Hexyl-2-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12br). Synthesized by procedure 2B with 2.12br-int2 to yield 2.12br in 14% as a yellow solid:

$^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.14 (s, 1H), 9.27 (s, 1H), 8.60 (d, $J = 8.3$ Hz, 1H), 7.42 – 7.34 (m, 2H), 2.71 (t, $J = 7.4$ Hz, 2H), 1.72 – 1.60 (m, 2H), 1.39 – 1.28 (m, 6H), 0.87 (t, $J = 7.0$ Hz, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ rotamers -58.31 (d, $J = 1.9$ Hz), -58.39 (d, $J = 1.8$ Hz);

$^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.8, 150.1, 145.3, 142.5, 140.5 (q, $J_{CF} = 2.4$ Hz), 128.8, 128.5, 123.7, 121.8, 121.7 (q, $J_{CF} = 258.0$ Hz), 35.9, 32.4, 32.1, 29.6, 23.3, 14.4; HRMS (ESI+) m/z calc’d. for C$_{17}$H$_{19}$F$_3$N$_5$O$_3$ (M+H)$^+$ 398.1434, found 398.1441.

6-((4-(2-Hydroxyethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bs). A round-bottom flask containing 2.11 (0.200 g, 1.05 mmol) was evacuated and refilled with N$_2$ (3x). The flask was cooled in an ice bath and the solid was sequentially diluted with anhydrous THF (2 mL), a mixture of 2-(4-aminophenyl)ethanol-1-ol (0.130 g, 0.948 mmol) in THF (2 mL), and Et$_3$N (0.15 mL, 1.1 mmol). The mixture, cooled in an ice bath, was stirred for 1.5 h, diluted with EtOAc,
filtered through a SiO$_2$ plug (EtOAc) to remove the salts, and concentrated to a residue. The residue was diluted with a solution of KOH (320 mg, 5.70 mmol) in H$_2$O (4 mL) and THF (2 mL). The resulting solution was stirred at rt for 1 h, acidified with 1 M aq. HCl, and extracted with EtOAc. The organic layer was washed with brine, dried (Na$_2$SO$_4$), and concentrated to a pale yellow residue (0.150 g). The residue was purified by chromatography on SiO$_2$ (gradient: 0-5% MeOH/CH$_2$Cl$_2$) to yield product (0.135 g) as an off-white solid at ca. 90% purity by NMR. The solid was diluted with EtOAc and then hexanes for a final mix of ca. 70% EtOAc/hexanes. The solid was filtered, rinsed with hexanes, and collected to afford **2.12bs** (0.106 g, at 95% purity by NMR, 39% yield) as a pale yellow solid: $^1$H NMR ((CD$_3$)$_2$SO, 400 MHz) δ 13.3 (br s, 1H), 10.2 (s, 1H), 7.91 – 7.88 (m, 2H), 7.26 – 7.23 (m, 2H), 4.66 (br s, 1H), 3.60 (t, $J = 7.1$ Hz, 2H), 2.72 (t, $J = 7.1$ Hz, 2H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.7, 151.0, 150.5, 145.1, 137.7, 136.6, 130.3, 122.1, 63.8, 39.8.; HRMS (ESI$^-$) m/z calc’d. for C$_{12}$H$_{10}$N$_5$O$_3$ (M-H) 272.0789, found 272.0796.

6-((2-Methyl-5-(trifluoromethyl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bt). Synthesized by procedure **2B** to yield **2.12bt** in 17% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.98 (s, 1H), 9.34 (s, 1H), 8.45 – 8.36 (m, 1H), 7.62 – 7.50 (m, 2H), 2.49 (s, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -62.87 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 101 MHz) δ 152.7, 151.3, 149.5, 144.3, 136.4, 136.3, 131.5, 128.3 (q, $J_{CF} = 32.4$ Hz), 124.3 (q, $J_{CF} = 271.2$ Hz), 122.5 (q, $J_{CF} = 3.9$ Hz), 120.7 (q, $J_{CF} = 4.1$ Hz), 17.0; HRMS (ESI$^+$) m/z calc’d. for C$_{12}$H$_5$F$_3$N$_5$O$_2$ (M+H)$^+$ 312.0702, found 312.0700.

6-((2-Methyl-4-(trifluoromethoxy)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bu). Synthesized by procedure **2A** to yield **2.12bu** in 42% as a pale yellow crystalline solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.08 (br s, 1H), 9.23 (s, 1H), 8.09 (d, $J = 8.8$ Hz, 1H), 7.32 (d, $J = 2.8$ Hz, 1H), 7.29 (dd, $J = 8.7$, 2.8 Hz, 1H), 2.44 (s, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -
58.62 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.7, 152.1, 150.5, 147.4 (q, $J_{CF} = 1.7$ Hz), 145.2, 135.5, 135.4, 126.7, 123.9, 121.5 (q, $J_{CF} = 255.5$ Hz), 119.8, 17.9; HRMS (ESI$^+$) $m/z$ calc’d. for C$_{12}$H$_3$F$_3$N$_5$O$_3$ (M+H)$^+$ 328.0652, found 328.0650.

2-((6-Hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)phenyl)-2-methylpropanenitrile (2.12bv). Synthesized by procedure 2A to yield 2.12bv in 78% as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.06 (s, 1H), 9.61 (s, 1H), 8.20 – 8.15 (m, 2H), 7.66 – 7.61 (m, 2H), 1.76 (s, 6H); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 153.6, 151.3, 150.4, 145.1, 139.4, 138.1, 126.6, 125.1, 122.5, 37.6, 29.2; HRMS (ES$^+$) $m/z$ calc’d. for C$_{14}$H$_{13}$N$_6$O$_2$ (M+H)$^+$ 297.1095, found 297.1092.

1-((6-Hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)phenyl)ethan-1-one (2.12bw). A round-bottom flask containing 2.11 (0.200 g, 1.05 mmol), 4-aminoacetophenone (0.130 g, 0.962 mmol), and K$_2$CO$_3$ (0.200 g, 1.45 mmol) was diluted with acetone/H$_2$O (9:1, 3 mL) and the resulting dark mixture was stirred at room temperature. After 2 h, the mixture was diluted with a solution of KOH in H$_2$O (0.320 g in 4 mL) and stirring was continued for an additional 30 min. The mixture was acidified with 1 M HCl and extracted with EtOAc. The organic layer was washed with brine, dried (MgSO$_4$), and concentrated to a yellow residue. The residue was purified by chromatography on SiO$_2$ (gradient: 0 – 2% MeOH/CH$_2$Cl$_2$) to afford 2.12bw (93% purity using HPLC condition 1) in 19% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.13 (br s, 1H), 9.73 (s, 1H), 8.28 (d, $J = 8.4$ Hz, 2H), 8.09 (d, $J = 8.5$ Hz, 2H), 2.60 (s, 3H); $^{13}$C NMR ((CD$_3$)$_2$CO, 126 MHz) δ 196.7, 153.5, 151.4, 150.2, 145.2, 142.7, 134.5, 130.2, 121.5, 26.6; HRMS (ES$^+$) $m/z$ calc’d. for C$_{12}$H$_{10}$N$_5$O$_3$ (M+H)$^+$ 272.0778, found 272.0793.

6-((Isopropylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bx). Synthesized by procedure 2B to yield 2.12bx in 60% as a tan solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.44 (s, 1H), 7.59 (s,
1H), 4.56 – 4.11 (m, 1H), 1.31 (d, J = 6.6 Hz, 6H); 13C NMR ((CD3)2CO, 101 MHz) δ 153.3, 152.5, 150.9, 144.8, 44.0, 21.7; HRMS (ESI+) m/z calc’d. for C7H10N5O2 (M+H)+ 196.0829, found 196.0826.

6-(Octylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12by). A round-bottom flask containing 2.11 (0.200 g, 1.05 mmol) and K2CO3 (0.200 g, 1.45 mmol) in acetone/H2O (9:1, 3 mL) was slowly diluted with a solution of n-octylamine (0.125 g, 0.967 mmol) in acetone/H2O (9:1, 1 mL) and the resulting dark mixture was stirred at rt. After 2 h, the mixture was diluted with a solution of KOH in H2O (0.320 g in 4 mL) and stirring was continued for an additional 30 min. The mixture was acidified with 1 M HCl and extracted with EtOAc. The organic layer was washed with brine, dried (MgSO4), and concentrated to a brown residue. The residue was purified by chromatography on SiO2 (gradient: 0.5 – 2% MeOH/CH2Cl2) to yield a crude brown solid. The solid was dissolved in EtOAc and extracted with 2-3 M NaOH. The aqueous layer was acidified with aq. HCl and extracted with EtOAc (3x). The organic layer was washed with brine, dried (MgSO4), and concentrated to yield 2.12by (9%) as an off-white solid: 1H NMR ((CD3)2SO, 500 MHz) δ 13.05 (s, 1H), 8.73 (t, J = 6.1 Hz, 1H), 3.36 (q, J = 6.8 Hz, 2H), 1.58 (p, J = 7.2 Hz, 2H), 1.33–1.20 (m, 10H), 0.85 (t, J = 6.7 Hz, 3H); 13C NMR ((CD3)2SO, 125 MHz) δ 152.8, 152.7, 150.2, 144.4, 40.7, 31.2, 28.7, 28.6, 26.4, 22.1, 13.9; HRMS (ESI+) m/z calc’d. for C12H20N5O2 (M+H)+ 266.1612, found 266.1628.

6-(Phenethylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12bz). Synthesized by procedure 2A to yield 2.12bz in 20% as a colorless solid: 1H NMR ((CD3)2CO, 500 MHz) δ 11.80 (br s, 1H), 8.01 (s, 1H), 7.33-7.17 (m, 5H), 3.85-3.77 (m, 2H), 3.07-2.99 (m, 2H); 13C NMR ((CD3)2CO, 125 MHz) δ 153.6, 153.3, 150.9, 145.0, 140.0, 129.7, 129.3, 127.2, 43.4, 35.0; HRMS (ESI+) m/z calc’d. for C12H20N5O2 (M-H)- 256.0840, found 256.0845.
6-((3,3,3-Trifluoropropyl)amino)-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-\textit{ol} \ (2.12\textit{ca}). Synthesized by procedure \textit{2A} to yield \textit{2.12ca} (94\% purity using HPLC condition 1) in 66\% yield as a light yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 11.82 (br s, 1H), 8.20 (s, 1H), 3.86 (q, $J$ = 6.7 Hz, 2H), 2.72 (qt, $J$ = 11.1, 7.0 Hz, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -65.98 – -66.05 (m, 3 F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) $\delta$ 153.8, 153.2, 150.7, 145.1, 127.6 (q, $J_{CF}$ = 276.1 Hz), 35.4 (q, $J_{CF}$ = 4.0 Hz), 32.7 (q, $J_{CF}$ = 27.8 Hz); HRMS (ESI$^+$) m/z calc’d. for C$_7$H$_4$F$_3$N$_5$O$_2$ (M-H) $^{\text{248.0401}}$, found 248.0413.

6-((4-Methylbenzyl)amino)-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-\textit{ol} \ (2.12\textit{cb}). A round-bottom flask containing \textit{2.11} (0.200 g, 1.05 mmol) and K$_2$CO$_3$ (0.200 g, 1.45 mmol) in acetone/H$_2$O (9:1, 3 mL) was slowly diluted with a solution of \textit{p}-tolylmethanamine (0.120 g, 0.990 mmol) in acetone/H$_2$O (9:1, 1 mL) and the resulting dark mixture was stirred at rt. After 1.5 h, the mixture was diluted with a solution of KOH in H$_2$O (0.320 g in 4 mL) and stirring was continued for an additional 30 min. The mixture was acidified with 1M HCl and extracted with EtOAc. The organic layer was washed with brine, dried (MgSO$_4$), and concentrated to a brown residue. The residue was purified by chromatography on SiO$_2$ (0.7\% MeOH/CH$_2$Cl$_2$) to yield \textit{2.12cb} in 29\% as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 11.76 (br s, 1H), 8.39 (s, 1H), 7.33 (d, $J$ = 7.7 Hz, 2H), 7.14 (d, $J$ = 7.7 Hz, 2H), 4.73 (d, $J$ = 6.4 Hz, 2H), 2.29 (s, 3H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) $\delta$ 153.6, 153.5, 150.9, 145.1, 137.7, 135.8, 129.9, 128.8, 45.0, 21.1; HRMS (ESI$^+$) m/z calc’d. for C$_{12}$H$_{12}$N$_5$O$_2$ (M+H)$^+$ 258.0986, found 258.0993.

6-((4-(Trifluoromethoxy)benzyl)amino)-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-\textit{ol} \ (2.12\textit{cc}). Synthesized by procedure \textit{2A} to yield \textit{2.12cc} in 36\% as a colorless solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 11.77 (br s, 1H), 8.59 (s, 1H), 7.59 (app d, $J$ = 8.6 Hz, 2H), 7.29 (d, $J$ = 8.2 Hz, 2H), 4.83 (d, $J$ = 6.5 Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -58.65 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO,
125 MHz) δ 153.9, 153.5, 150.9, 149.1 (q, $J_{CF} = 1.6$ Hz), 145.2, 138.4, 130.6, 121.9, 121.4 (q, $J_{CF} = 255.2$ Hz), 44.5; HRMS (ESI) $m/z$ calc'd. for C$_{12}$H$_7$F$_3$N$_5$O$_3$ (M-H)$^-$ 326.0506, found 326.0492.

6-(Benzhydrylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12cd). Synthesized by procedure 2A to yield 2.12cd in 63% as a colorless solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 11.92 (br s, 1H), 8.30 (d, $J = 8.7$ Hz, 1H), 7.48 – 7.45 (m, 4H), 7.40 – 7.35 (m, 4H), 7.33 – 7.28 (m, 2H), 6.58 (d, $J = 8.6$ Hz, 1H); $^{13}$C NMR ((CD$_3$)$_2$CO, 100 MHz) δ 153.4, 152.9, 150.8, 145.2, 141.8, 129.5, 128.6, 128.4, 59.4; HRMS (ESI) $m/z$ calc’d. for C$_{17}$H$_{12}$N$_5$O$_2$ (M-H)$^-$ 318.0996, found 318.1003.

6-(Adamantan-1-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ce). Synthesized by procedure 2A at 75 °C to yield 2.12ce in 55% as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 11.83 (br s, 1H), 6.97 (s, 1H), 2.30 – 2.24 (m, 6H), 2.16 – 2.12 (m, 3H), 1.80 – 1.72 (m, 6H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.6, 152.0, 150.4, 144.7, 54.2, 41.0, 36.9, 30.3; HRMS (ESI) $m/z$ calc’d. for C$_{14}$H$_{16}$N$_5$O$_2$ (M-H)$^-$ 286.1309, found 286.1311.

6-(Adamantan-2-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12cf). Synthesized by procedure 2A with a reverse-phase HPLC purification using 0.1% TFA MeCN/H$_2$O solvent system to yield 2.12cf in 5% as a colorless solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 11.57 (br s, 1H), 7.38 (s, 1H), 4.27 – 4.24 (m, 1H), 2.18 – 2.16 (m, 2H), 2.00 – 1.88 (m, 8H), 1.83 – 1.81 (m, 2H), 1.74 – 1.70 (m, 2H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.9, 152.6, 151.0, 145.3, 56.2, 38.0, 37.6, 32.4, 31.9, 28.1; HRMS (ESI$^+$) $m/z$ calc’d. for C$_{14}$H$_{18}$N$_5$O$_2$ (M+H)$^+$ 288.1455, found 288.1458.

6-(3,5-Dimethyladamantan-1-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12cg). Synthesized by procedure 2A at 75 °C to yield 2.12cg in 38% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 11.84 (br s, 1H), 6.98 (s, 1H), 2.22 (hept, $J = 3.2$ Hz, 1H), 2.13 – 2.08 (m, 2H), 1.93 – 1.85 (m, 4H), 1.47 (dt, $J = 12.2$, 2.7 Hz, 2H), 1.37 (dt, $J = 12.3$, 2.8 Hz, 2H), 1.23 (qt, $J = 12.4$, 2.1 Hz, 2H), 0.90 (s, 6H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.6, 152.0, 150.4,
144.7, 55.8, 51.1, 46.9, 43.2, 39.5, 31.0, 30.5; HRMS (ESI) m/z calc’d. for C_{16}H_{20}N_{5}O_{2} (M-H)^{-} 314.1622, found 314.1616.

6-(3-Hydroxyadamantan-1-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ch). Synthesized by procedure 2A at 75 °C to yield 2.12ch in 30% as an off-white solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 11.84 (br s, 1H), 7.04 (s, 1H), 3.81 (br s, 1H), 2.31 – 2.28 (m, 2H), 2.22 – 2.11 (m, 6H), 1.77 – 1.68 (m, 4H), 1.67 – 1.57 (m, 2H); \(^1^3\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 153.6, 152.0, 150.4, 144.7, 68.7, 56.6, 48.6, 45.0, 40.0, 35.7, 31.5; HRMS (ESI\(^{+}\)) m/z calc’d. for C\(_{16}\)H\(_{20}\)N\(_5\)O\(_2\) (M+H\(^{+}\)) 314.1622, found 314.1616.

6-(Adamantan-1-yl)ethyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (2.12ci). Synthesized by procedure 2A at 75 °C to yield 2.12ci in 8% as a beige solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 11.84 (s, 1H), 7.27 (d, \(J\) = 9.7 Hz, 1H), 4.05 (dq, \(J\) = 10.0, 6.9 Hz, 1H), 1.99 (p, \(J\) = 3.2 Hz, 3H), 1.76 – 1.62 (m, 12H), 1.20 (d, \(J\) = 6.9 Hz, 3H); \(^1^3\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 153.6, 153.3, 151.0, 144.9, 55.9, 39.1, 37.7, 37.3, 29.3, 13.9; HRMS (ESI\(^{+}\)) m/z calc’d. for C\(_{16}\)H\(_{20}\)N\(_5\)O\(_2\) (M-H)^{-} 314.1622, found 314.1618.

6-Methoxy-N-(4-(trifluoromethoxy)phenyl)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-amine (2.13). Synthesized by procedure 2A to yield 2.13 in 60% as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 9.70 (s, 1H), 8.19 – 8.10 (m, 2H), 7.45 – 7.36 (m, 2H), 4.23 (s, 3H); \(^1^9\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -58.78 (s, 3F); \(^1^3\)C NMR ((CD\(_3\))\(_2\)CO, 126 MHz) \(\delta\) 156.4, 151.7, 150.6, 147.7, 146.5 (q, \(J\)\(_{CF}\) = 2.3 Hz), 137.8, 124.1, 122.4, 121.5 (q, \(J\)\(_{CF}\) = 255.2 Hz), 56.5; HRMS (ESI\(^{+}\)) m/z calc’d. for C\(_{12}\)H\(_9\)F\(_3\)N\(_5\)O\(_3\)\(^{+}\) 328.0637, found 328.0652.

3-((2-Fluorophenyl)amino)pyrazin-2-ol (2.16a). Synthesized by procedure 2C to yield 2.16a in 32% as an off white solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 10.98 (s, 1H), 8.79 – 8.68 (m, 1H), 8.42 (s, 1H), 7.27 – 7.14 (m, 2H), 7.08 – 7.00 (m, 1H), 6.97 (d, \(J\) = 4.5 Hz, 1H), 6.91 (d, \(J\) = 4.5 Hz, 2H), 6.80 (d, \(J\) = 8.4 Hz, 1H), 6.70 (d, \(J\) = 8.7 Hz, 1H), 6.62 (s, 1H), 4.14 (s, 3H); \(^1^3\)C NMR ((CD\(_3\))\(_2\)CO, 126 MHz) \(\delta\) 156.3, 150.5, 147.7, 146.5 (q, \(J\)\(_{CF}\) = 2.3 Hz), 138.5, 124.1, 122.4, 121.5 (q, \(J\)\(_{CF}\) = 255.2 Hz), 56.6, 48.6, 45.0, 40.0, 35.7, 31.5; HRMS (ESI\(^{+}\)) m/z calc’d. for C\(_{16}\)H\(_{20}\)N\(_5\)O\(_2\) (M+H)^{+} 314.1622, found 314.1616.
Hz, 1H); \(^{19}\text{F NMR}\) ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -134.06 – -134.15 (m, 1F); \(^{13}\text{C NMR}\) ((CD\(_3\))\(_2\)CO, 101 MHz) \(\delta\) 153.3 (d, \(J_{CF} = 241.9\) Hz), 149.7, 139.1, 136.5, 125.3 (d, \(J_{CF} = 3.9\) Hz), 123.4 (d, \(J_{CF} = 7.7\) Hz), 121.5, 120.7 (d, \(J_{CF} = 1.6\) Hz), 116.9, 115.4 (d, \(J_{CF} = 19.1\) Hz); HRMS (ESI\(^{+}\)) calc’d for C\(_{10}\)H\(_9\)FN\(_3\)O (M+H)\(^{+}\) 206.0724, found 206.0716.

3-((4-Decylphenyl)amino)pyrazin-2-ol (2.16b). Synthesized by procedure 2C to yield 2.16b in 33% as an off white solid: \(^1\text{H NMR}\) (CDCl\(_3\), 400 MHz) \(\delta\) 12.16 (s, 1H), 8.04 (s, 1H), 7.68 (d, \(J = 8.1\) Hz, 2H), 7.17 (d, \(J = 8.1\) Hz, 2H), 7.09 (d, \(J = 4.4\) Hz, 1H), 6.67 (d, \(J = 4.4\) Hz, 1H), 2.58 (t, \(J = 7.7\) Hz, 2H), 1.60 (p, \(J = 7.2\) Hz, 2H), 1.40 – 1.19 (m, 14H), 0.88 (t, \(J = 6.6\) Hz, 3H); \(^{13}\text{C NMR}\) (CDCl\(_3\), 101 MHz) \(\delta\) 153.1, 148.9, 138.0, 136.5, 129.0, 123.4, 119.4, 114.0, 35.5, 32.0, 31.7, 29.8, 29.8, 29.7, 29.5, 29.4, 22.8, 14.3; HRMS (ESI\(^{+}\)) calc’d for C\(_{20}\)H\(_{30}\)N\(_3\)O (M+H)\(^{+}\) 328.2383, found 328.2383.

3-((4-(Trifluoromethoxy)phenyl)amino)pyrazin-2-ol (2.16c). Synthesized by procedure 2C to yield 2.16c to yield in 12% as an off white solid: \(^1\text{H NMR}\) ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 10.90 (s, 1H), 8.77 (s, 1H), 8.21 – 8.13 (m, 2H), 7.33 – 7.26 (m, 2H), 6.94 (d, \(J = 4.4\) Hz, 1H), 6.87 (d, \(J = 4.4\) Hz, 1H); \(^{19}\text{F NMR}\) ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -58.87 (s, 3F); \(^{13}\text{C NMR}\) (101 MHz, Acetone-d\(_6\)) \(\delta\) 152.4, 150.0, 144.3 – 144.2 (m), 140.1, 122.3, 121.6 (q, \(J_{CF} = 254.3\) Hz), 121.5, 120.9, 116.6; HRMS (ESI\(^{+}\)) calc’d for C\(_{11}\)H\(_9\)F\(_3\)N\(_3\)O\(_2\) (M+H)\(^{+}\) 272.0641, found 272.0627.

3-((3,5-Bis(trifluoromethyl)phenyl)amino)pyrazin-2-ol (2.16d). Synthesized by procedure 2C to yield 2.16d to yield in 36% as a yellow solid: \(^1\text{H NMR}\) ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 11.02 (s, 1H), 9.18 (s, 1H), 8.86 – 8.79 (m, 2H), 7.68 – 7.56 (m, 1H), 7.02 (d, \(J = 4.5\) Hz, 1H), 6.97 (d, \(J = 4.5\) Hz, 1H); \(^{19}\text{F NMR}\) (376 MHz, Acetone-\(d_6\)) \(\delta\) -63.53 (s, 6F); \(^{13}\text{C NMR}\) ((CD\(_3\))\(_2\)CO, 101 MHz) \(\delta\) 152.2, 150.0, 142.8, 132.3 (q, \(J_{CF} = 33.2\) Hz), 125.9 (q, \(J_{CF} = 272.3\) Hz), 121.1, 119.8 – 119.1
(m), 117.9, 115.5 – 115.1 (m); HRMS (ESI⁺) calc’d for C₁₂H₈F₆N₃O (M+H)⁺ 324.0566, found 324.0556.

3-((4-(Trifluoromethyl)phenyl)amino)pyrazin-2-ol (2.16e). Synthesized by procedure 2C to yield 2.16e to yield in 37% as an off white solid: ¹H NMR ((CD₃)₂CO, 400 MHz) δ 10.98 (s, 1H), 8.87 (s, 1H), 8.30 – 8.21 (m, 2H), 7.73 – 7.62 (m, 2H), 6.98 (d, J = 4.5 Hz, 1H), 6.92 (d, J = 4.4 Hz, 1H); ¹⁹F NMR ((CD₃)₂CO, 376 MHz) δ -62.14 (s, 3F); ¹³C NMR ((CD₃)₂CO, 101 MHz) δ 152.3, 150.0, 144.3 (d, J⁰CF = 1.8 Hz), 126.7 (q, J⁰CF = 4.1 Hz), 125.7 (q, J⁰CF = 270.7 Hz), 123.8 (q, J⁰CF = 32.3 Hz), 121.3, 119.5, 117.2; HRMS (ESI⁺) calc’d for C₁₁H₉F₃N₃O (M+H)⁺ 256.0692, found 256.0698.

3-((3-(Trifluoromethyl)phenyl)amino)pyrazin-2-ol (2.16f). Synthesized by procedure 2C to yield 2.16f to yield in 35% as a brown solid: ¹H NMR ((CD₃)₂CO, 400 MHz) δ 10.86 (s, 1H), 8.87 (s, 1H), 8.61 (s, 1H), 8.22 (dd, J = 8.3, 2.2 Hz, 1H), 7.54 (t, J = 8.0 Hz, 1H), 7.33 (d, J = 7.7, 1H), 6.98 (d, J = 4.4 Hz, 1H), 6.91 (d, J = 4.5 Hz, 1H); ¹⁹F NMR ((CD₃)₂CO, 376 MHz, Acetone-d₆) δ -63.13 (s, 3F); ¹³C NMR ((CD₃)₂CO, 101 MHz) δ 152.4, 150.1, 141.7, 131.2 (d, J⁰CF = 31.9 Hz), 130.3, 125.4 (q, J⁰CF = 271.4 Hz), 123.2 (d, J⁰CF = 1.5 Hz), 121.5 – 121.3 (m), 119.1 (q, J⁰CF = 4.3 Hz), 116.9, 115.9 (q, J⁰CF = 4.2 Hz); HRMS (ESI⁺) calc’d for C₁₁H₉F₃N₃O (M+H)⁺ 256.0692, found 256.0698.

3-((3’-methoxy-[1,1'-biphenyl]-4-yl)amino)pyrazin-2-ol (2.16g). Synthesized by procedure 2C to yield 2.16g to yield in 30% as a brown solid: ¹H NMR ((CD₃)₂SO, 500 MHz) δ 11.97 (s, 1H), 9.20 (s, 1H), 8.17 – 8.02 (m, 2H), 7.69 – 7.53 (m, 2H), 7.35 (t, J = 7.9 Hz, 1H), 7.26 – 7.20 (m, 1H), 7.18 (t, J = 2.1 Hz, 1H), 6.92 (d, J = 4.3 Hz, 1H), 6.89 (dd, J = 8.2, 2.5 Hz, 1H), 6.82 (d, J = 4.3 Hz, 1H), 3.82 (s, 3H); ¹³C NMR ((CD₃)₂SO, 101 MHz) δ 159.7, 151.4, 148.9, 141.4, 139.6,
133.4, 129.9, 126.7, 120.5, 119.4, 119.3, 118.5, 115.7, 112.3, 111.7, 55.1; HRMS (ESI+) calc’d for C_{17}H_{16}N_{3}O_{2} (M+H)^{+} 294.1237, found 294.1236

3-((4'-Fluoro-[1,1'-biphenyl]-4-yl)amino)pyrazin-2-ol (2.16h). Synthesized by procedure 2C to yield 2.16h to yield in 16% as an off brown solid: \(^1\)H NMR ((CD\(_3\))\(_2\)SO, 400 MHz) \(\delta\) 11.97 (s, 1H), 9.21 (s, 1H), 8.12 – 8.03 (m, 2H), 7.69 – 7.61 (m, 2H), 7.62 – 7.52 (m, 2H), 7.33 – 7.17 (m, 2H), 6.90 (m, 1H), 6.80 (m, 1H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)SO, 376 MHz) \(\delta\) -116.47 (tt, \(J = 8.9, 5.4\) Hz, 1F); \(^{13}\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 161.5 (d, \(J_{CF} = 243.5\) Hz), 151.5, 148.9, 139.4, 136.4 (d, \(J_{CF} = 3.0\) Hz), 132.5, 128.0 (d, \(J_{CF} = 8.0\) Hz), 126.6, 120.5, 119.5, 115.7, 115.6 (d, \(J_{CF} = 21.4\) Hz); HRMS (ESI+) calc’d for C\(_{16}\)H\(_{13}\)FN\(_3\)O (M+H)^{+} 282.1037, found 282.1037.

4.3 Synthetic Procedures and Characterization of Compounds for Chapter 3

4.3.1 STAM Mouse Study.

The STAM mouse model was performed by SMC Laboratories, Inc. (Tokyo, Japan). C57BL/6 mice (14-day-pregnant female) were obtained from Japan SLC, Inc. (Japan). All animals used in the study were housed and cared for in accordance with the Japanese Pharmacological Society Guidelines for Animal Use. The animals were maintained in a SPF facility under controlled conditions of temperature (23 ± 2 °C), humidity (45 ± 10%), lighting (12 h artificial light and dark cycles; light from 8:00 to 20:00), and air exchange. A high pressure was maintained in the experimental room to prevent contamination of the facility. NASH was induced in 24 male mice by a single subcutaneous injection of 200 μg streptozotocin (Sigma-Aldrich, USA) solution 2 days after birth and feeding with HFD after 4 weeks of age. Compounds were administered in diet by premixing high-fat diet (HFD, 57 kcal % fat, catalog no. HFD32, CLEA Japan Inc., Japan) at SMC Laboratories. Resmetirom was purchased from Focus Bioscience (HY-12216). 9b and Resmetirom were administered from 6 to 10 weeks of age as food admixture in HFD at doses...
equivalent to 60 and 3 mg kg⁻¹ d⁻¹, respectively. The viability, clinical signs and behavior were monitored daily. Body weight was recorded daily before treatment, and food intake was measured twice weekly for each cage (4 mice per cage). Mice were observed for significant clinical signs of toxicity, moribundity and mortality approximately 60 minutes after each administration. The animals were sacrificed at 10 weeks of age (day 28) by exsanguination through direct cardiac puncture under isoflurane anesthesia (Pfizer Inc.). For liver samples, the left lateral lobe was collected and cut into six pieces. Two pieces of left lateral lobe, left and right medial lobes, and caudate lobe were snap frozen in liquid nitrogen and stored at −80°C. The other two pieces of left lateral lobe were fixed in Bouin’s solution and then embedded in paraffin. Samples were stored at room temperature for histology. The remaining pieces of left lateral lobe were embedded in O.C.T. compound and quick frozen in liquid nitrogen. Samples were stored at −80°C. The right lobe was snap frozen in liquid nitrogen and stored at −80°C for liver biochemistry. Liver total lipid-extracts were obtained by Folch’s method. Briefly, liver samples were homogenized in chloroform–methanol (2:1, v/v) and incubated overnight at room temperature. After washing with chloroform–methanol–water (8:4:3, v/v/v), the extracts were evaporated to dryness and dissolved in isopropanol. Liver triglyceride content was measured by triglyceride E-test (Wako Pure Chemical Industries, Ltd., Japan). For histopathology staining, sections were cut from paraffin blocks of liver tissue prefixed in Bouin’s solution and stained with Lillie-Mayer’s hematoxylin (Muto Pure Chemicals Co., Ltd., Japan) and eosin solution (Wako Pure Chemical Industries). NAFLD activity score (NAS) was calculated according to the criteria of Kleiner. To visualize collagen deposition, Bouin’s fixed liver sections were stained using Picrosirius Red solution (Waldeck, Germany). For quantitative analysis of fibrosis areas, bright field images of Sirius Red stained sections were captured around the central vein using a digital camera (DFC295; Leica,
Germany) at 200-fold magnification, and the positive areas in 5 fields/section were measured using ImageJ software (National Institutes of Health, USA).

4.3.2 Plasma Assays for STAM Model.

Non-fasting blood glucose was measured in whole blood using Stat Strip glucose meter (NIPRO Corporation, Japan). For plasma biochemistry, non-fasting blood was collected in polypropylene tubes with anticoagulant (Novo-Heparin, Mochida Pharmaceutical Co. Ltd., Japan) and centrifuged at 1000g for 15 min at 4 °C. The supernatant was collected and stored at −80°C until use. Plasma ALT level was measured by FUJI DRI-CHEM 7000 (Fujifilm, Japan). Plasma triglyceride was measured by a colorimetric assay through reaction with GPO reagent (Pointe Scientific T7532) according to the manufacturer’s instructions. A standard curve was constructed using serial dilutions of glycerol standard (Sigma G7793). Samples were incubated with GPO at 37 °C for 5–20 min until absorbance values at 500 nm had stabilized, and plasma triglyceride concentrations were determined through interpolation from the standard curve. Plasma cholesterol was measured by a colorimetric assay using Infinity cholesterol liquid stable reagent (Thermo Fisher TR13421) according to the manufacturer’s instructions. Samples were incubated with Infinity cholesterol reagent at 37 °C for 5–20 min until absorbance values at 500 nm had stabilized. Absorbance at 660 nm was subtracted from absorbance at 500 nm to correct for background. A standard curve was constructed using serial dilutions of cholesterol standard (Pointe Scientific C7509), and sample plasma cholesterol concentrations were determined through interpolation from the standard curve. Plasma AST was measured using Infinity AST (GOT) liquid stable reagent (Thermo Fisher TR70121) according to the manufacturer’s instructions. Briefly, samples were incubated with the Infinity AST reagent at 37 °C and absorbance was measured at 340 nm every minute for 20 min. Activity in U/L was calculated by multiplying the change in absorbance
per minute by a correction factor, defined as total volume of reaction divided by the product of the molar absorption coefficient of NADH, the sample volume added, and the path length of absorption.

4.3.3 Statistical Analysis.

All data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism (version 8.4.3; GraphPad software), where the threshold for significance was designated as p<0.05, compared to vehicle. For normally distributed data, differences between groups were examined using a one-way analysis of variance (ANOVA) with Dunnett’s post hoc test for multiple comparisons. For nonparametric data, the Kruskal–Wallis test was conducted with Dunn’s post hoc test for multiple comparisons.

4.3.4 General Synthetic Procedures.

**General Procedure 3.1 (Direct Substitution with Biphenylamines Method):** In a screw-cap vial or round-bottom flask, the requisite amine (0.70 – 0.98 mmol) was added to a stirring mixture of dichloro 3.8 (0.200 g, 1.05 mmol) in anhydrous THF (or acetone where indicated) (0.1 – 0.2 M). Then, Et₃N (1.1 mmol or 2.2 mmol when an amine salt is used) was added and the resulting dark mixture was stirred at room temperature (unless otherwise indicated) for 2 – 20 h. The reaction mixture was diluted with an aqueous solution of KOH (6 equiv) and stirring at room temperature was continued for 0.5 – 2 h. The mixture was acidified with 1 M aqueous HCl and extracted with EtOAc. The organic layer was washed with brine, dried (Na₂SO₄), and concentrated to a residue. The residue was purified by chromatography on SiO₂ using a MeOH/CH₂Cl₂ or EtOAc/hexanes solvent system to yield the desired product. When additional purification was needed, the solid was dissolved in a minimal amount of hot acetone, allowed to
cool to room temperature, and precipitated by the addition of hexanes. The precipitate was filtered, rinsed with hexanes, and collected to yield the desired product 3.9.

**General Procedure 3.2 (Suzuki Cross-Coupling Method):** Halogenated (Br, I) aniline was installed onto 3.8 following general procedure 3.1. The resulting compound (0.616 mmol) was added to a reaction tube containing a stir bar followed by necessary aryl boronic acid (0.616 mmol), Na₂CO₃ (1.84 mmol), and Pd(dppf)Cl₂·CH₂Cl₂ (1 mol%). The tube was sealed, evacuated, and filled with N₂ (3x). Deoxygenated 1,4-dioxane:H₂O (2:1, 3 mL) was then added and the reaction was heated to 90 °C for 16 h. The reaction was allowed to cool to room temperature and the resulting biphasic mixture was condensed under reduced pressure and loaded onto Celite. Compound was purified by column chromatography on SiO₂ (gradient: 1-5% MeOH/CH₂Cl₂) to yield the desired product 3.9 as a solid.

**General Procedure 3.3:** Aryl boronic acid or boronic acid pinacol ester (BPin) was installed onto 3.8 following general procedure 3.1. The resulting compound (0.616 mmol) was added to a reaction tube containing a stir bar followed by necessary aryl halide (1.0 equiv), Na₂CO₃ (2.3 equiv), and Pd(dppf)Cl₂·CH₂Cl₂ (1 mol%). The tube was sealed, evacuated, and filled with N₂ (3x). Deoxygenated 1,4-dioxane:H₂O (2:1, 3 mL) was then added and the reaction was heated to 90 °C for 16 h. The reaction was allowed to cool to room temperature and the resulting biphasic mixture was condensed under reduced pressure and loaded onto Celite. Compound was purified by column chromatography on SiO₂ (gradient: 1-5% MeOH/CH₂Cl₂) to yield the desired product 3.9 as a solid.

**General Procedure 3.4 (Modified Direct Substitution Method):** 5,6-Dichloro-[1,2,5]oxadiazolo[3,4-b]pyrazine 3.8 (1 equiv) was added to a round bottom flask containing a stir bar and dissolved in anhydrous THF. To the solution was added Et₃N (1.1 equiv), followed by
MeOH (1 equiv) in a dropwise fashion. The solution evolved into a slurry and was allowed to stir at room temperature for 30 min. Upon completion, the solvent was then removed under reduced pressure and crude material was purified by column chromatography on SiO₂ (gradient: 6% acetone/hexanes) to yield 5-chloro-6-methoxy-[1,2,5]oxadiazolo[3,4-b]pyrazine (70%). Next, the requisite amine (0.70 – 0.98 mmol) was added to a stirring mixture of methoxy chloro (0.200 g, 1.05 mmol) in anhydrous THF (or acetone where indicated) (0.1 – 0.2 M). Then, Et₃N (1.1 mmol or 2.2 mmol when an amine salt is used) was added and the resulting dark mixture was stirred at room temperature (unless otherwise indicated) for 2 – 20 h. The reaction mixture was diluted with an aqueous solution of KOH (6 equiv) and stirring at room temperature was continued for 0.5 – 2 h. The mixture was acidified with 1 M aqueous HCl and extracted with EtOAc. The organic layer was washed with brine, dried (Na₂SO₄), and concentrated to a residue. The residue was purified by chromatography on SiO₂ using a MeOH/CH₂Cl₂ or EtOAc/hexanes solvent system to yield the desired product. When additional purification was needed, the solid was dissolved in a minimal amount of hot acetone, allowed to cool to room temperature, and precipitated by the addition of hexanes. The precipitate was filtered, rinsed with hexanes, and collected to yield the desired product 3.9.

**General Procedure 3.5 (Preparation of Biaryl amine by Suzuki Cross-Coupling):** In a sealed vial, a mixture of the aryl boronic acid or aryl BPin (1.3 equiv), aryl halide (1.0 equiv), Pd(dppf)Cl₂·CH₂Cl₂ (1 – 5 mol%), and Na₂CO₃ (2.3 equiv) in deoxygenated dioxane/H₂O (2:1, 0.1 – 0.2 M) was stirred at 90 ºC under an atmosphere of N₂ overnight. The mixture was allowed to cool to room temperature, diluted with EtOAc, washed with brine, dried (Na₂SO₄), and concentrated to a residue. The residue was purified by chromatography on SiO₂ with a solvent system of EtOAc/hexanes to yield the desired biaryl aniline.
4.3.5 Characterizations.

6-((1,1'-Biphenyl)-4-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9a).

Synthesized by procedure 3.1 in acetone to yield 3.9a (157 mg, 53%) as a burnt-orange solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.01 (br s, 1H), 9.60 (s, 1H), 8.29 – 8.18 (m, 2H), 7.80 – 7.75 (m, 2H), 7.74 – 7.66 (m, 2H), 7.49 – 7.46 (m, 2H), 7.39 – 7.33 (m, 1H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.7, 151.2, 150.5, 145.2, 141.1, 138.1, 138.4, 138.1, 129.8, 128.2, 128.1, 127.5, 122.5; HRMS (ESI) m/z calcd for C$_{16}$H$_{10}$N$_5$O$_2$ (M-H) - 304.0840, found 304.0850.

6-((4'-Fluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9b).

Synthesized by procedure 3.1 using acetone to yield 3.9b (177 mg, 60%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.04 (br s, 1H), 9.58 (s, 1H), 8.24 – 8.21 (m, 2H), 7.76 – 7.73 (m, 4H), 7.25 – 7.22 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -117.30 – -117.38 (m, F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 163.3 (d, $J_{CF}$ = 244.6 Hz), 153.6, 151.1, 150.4, 145.1, 138.1, 137.5 (d, $J_{CF}$ = 3.3 Hz), 137.3, 129.4 (d, $J_{CF}$ = 8.2 Hz), 128.0, 122.5, 116.5 (d, $J_{CF}$ = 21.7 Hz); HRMS (ESI$^+$) m/z calcd for C$_{16}$H$_{11}$FN$_5$O$_2$ (M+H)$^+$ 324.0891, found 324.0895.

6-((4'-(Difluoromethoxy)-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9c).

Synthesized by procedure 3.3 to yield 3.9c (102 mg, 65%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.02 (br s, 1H), 9.61 (s, 1H), 8.26-8.22 (m, 2H), 7.80-7.74 (m, 4H), 7.32-7.27 (m, 2H), 7.05 (t, $J_{HF}$ = 74.3 Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -82.70 (d, $J_{HF}$ = 74.3, 2F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.7, 151.9 (t, $J_{CF}$ = 3.1 Hz), 151.1, 150.4, 145.2, 138.22, 138.16, 137.2, 129.1, 128.0, 122.5, 120.3, 117.5 (t, $J_{CF}$ = 257.2 Hz); HRMS (ESI$^+$) m/z calc’d. for C$_{17}$H$_{12}$F$_2$N$_5$O$_3$ (M+H)$^+$ 372.0903, found 372.0902.
6-((4’-(Trifluoromethoxy)-[1,1’-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9d).

Synthesized by procedure 3.3 to yield 3.9d (93 mg, 57%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.08 (br s, 1H), 9.63 (s, 1H), 8.28-8.25 (m, 2H), 7.87-7.83 (m, 2H), 7.82-7.78 (m, 2H), 7.46-7.43 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -58.57 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.6, 151.2, 150.4, 149.3 (q, $J_{CF} = 2.2$ Hz), 145.1, 140.3, 138.5, 136.8, 129.3, 128.2, 122.5, 122.3, 121.5 (q, $J_{CF} = 255.3$ Hz); HRMS (ESI$^+$) m/z calc’d for C$_{17}$H$_{11}$F$_3$N$_5$O$_3$ (M+H)$^+$ 390.0809, found 390.0821.

6-((4’-(Trifluoromethyl)-[1,1’-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9e).

Synthesized by procedure 3.2 to yield 3.9e (21 mg, 23% as a yellow solid: $^1$H NMR (500 MHz, (CD$_3$)$_2$CO) δ 12.15 (s, 1H), 9.66 (s, 1H), 8.35 – 8.27 (m, 2H), 7.95 (d, $J = 7.6$ Hz, 2H), 7.90 – 7.80 (m, 4H); $^{19}$F NMR (376 MHz, (CD$_3$)$_2$CO) δ -62.90 (s, 1F); $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) δ 153.6, 151.2, 150.3, 145.1, 144.8 (q, $J_{CF} = 1.2$ Hz), 138.9, 136.4, 129.4 (q, $J_{CF} = 32.7$ Hz), 128.4, 128.1, 127.6 (q, $J_{CF} = 274.5$ Hz), 126.6 (q, $J_{CF} = 4.0$ Hz), 122.5; HRMS (ESI$^+$) calc’d for C$_{17}$H$_{11}$F$_3$N$_5$O$_2$ (M+H)$^+$ 374.0859, found 374.0863.

4’-((6-Hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)-[1,1’-biphenyl]-4-carbonitrile (3.9f).

The requisite aniline was prepared by procedure 3.5 to yield 4’-amino-[1,1’-biphenyl]-4-carbonitrile in 81% as an off-white solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) δ 7.8 – 7.7 (m, 4H), 7.5 – 7.4 (m, 2H), 6.7 – 6.6 (m, 2H), 5.5 (s, 2H); $^{13}$C NMR (126 MHz, (CD$_3$)$_2$SO) δ 149.8, 145.1, 132.6, 127.8, 125.6, 124.8, 119.3, 114.2, 107.6; HRMS (ESI$^+$) calc’d for C$_{26}$H$_{21}$N$_4$ (2M+H)$^+$ 389.1761, found 389.1727.
Product 3.9f was synthesized by procedure 3.4 (192 mg, 70%) as a yellow solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) δ 13.4 (s, 1H), 10.42 (s, 1H), 8.22 – 8.19 (m, 2H), 7.92 (s, 4H), 7.86 – 7.83 (m, 2H); $^{13}$C NMR (126 MHz, (CD$_3$)$_2$SO) δ 153.0, 150.9, 149.6, 144.6, 143.9, 138.5, 134.1, 132.9, 127.4, 127.2, 122.1, 119.0, 109.7; HRMS (ESI$^+$) calc’d for C$_{17}$H$_{11}$N$_6$O$_2$ (M+H)$^+$ 331.0938, found 331.0930.

6-((4-(Benzo[d][1,3]dioxol-5-yl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9g).

The requisite aniline was prepared by procedure 3.5 to yield 4-(benzo[d][1,3]dioxol-5-yl)aniline in 61% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 7.32 – 7.28 (m, 2H), δ 7.03 (dd, $J = 1.9$, 0.5 Hz, 1H), 7.01 (dd, $J = 8.0$, 1.8 Hz, 1H), 6.85 (dd, $J = 8.0$, 0.5 Hz, 1H), 6.74 – 6.70 (m, 2H), 5.99 (s, 2 H), 4.70 (br s, 2H).

Product 3.9g was synthesized using acetone as the solvent by procedure 3.1 (227 mg, 61%) as a light brown solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.04 (br s, 1H), 9.58 (s, 1H), 8.21-8.18 (m, 2H), 7.71-7.69 (m, 2H), 7.82-7.78 (m, 2H), 7.22-7.19 (m, 2H), 6.95 (d, $J = 8.0$ Hz, 1H), 6.05 (s, 2H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.6, 151.1, 150.5, 149.3, 148.2, 145.1, 138.2, 137.7, 135.4, 127.8, 122.4, 121.1, 109.4, 107.9, 102.2; HRMS (ESI$^+$) m/z calc’d. for C$_{17}$H$_{10}$N$_5$O$_4$ (M-H)$^-$ 348.0738, found 348.0739.

6-((4-(2,2-Difluorobenzo[d][1,3]dioxol-5-yl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9h).

The requisite aniline was prepared by procedure 3.5 to yield 4-(2,2-difluorobenzo[d][1,3]dioxol-5-yl)aniline in 71% as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 7.44 – 7.43 (m, 1H), 7.39 – 7.33 (m, 3H), 7.28 – 7.25 (m, 1H), 6.77 – 6.73 (m, 2H), 4.84 (br, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -51.30 – -51.32 (m, 2F).
Product 3.9h was synthesized by procedure 3.1 (149 mg, 53%) as a burnt orange solid: $^1$H NMR ((CD$_3$)$_2$SO, 500 MHz) δ 13.31 (s, 1H), 10.39 (s, 1H), 8.15 (d, $J = 8.7$ Hz, 2H), 7.78 (d, $J = 1.8$ Hz, 1H), 7.74 (d, $J = 8.6$ Hz, 2H), 7.54 (dd, $J = 8.4$, 1.8 Hz, 1H), 7.49 (d, $J = 8.4$ Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$SO, 376 MHz) δ -51.19 (s, 2F); $^{13}$C NMR ((CD$_3$)$_2$SO, 125 MHz) δ 153.0, 150.8, 149.7, 144.6, 143.5, 142.1, 137.6, 136.7, 134.9, 131.3 (t, $J_{CF} = 252.8$ Hz), 127.0, 122.7, 122.1, 110.4, 108.4; HRMS (ESI$^-$) m/z calcd for C$_{17}$H$_8$F$_2$N$_5$O$_4$ (M-H)$^-$ 384.0550, found 384.0531.

6-((4-(Thiophen-2-yl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9i).

Synthesized by procedure 3.3 to yield 3.9i (87 mg, 66%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 12.07 (br s, 1H), 9.61 (s, 1H), 8.21-8.19 (m, 2H), 7.78-7.76 (m, 2H), 7.49 (d, $J = 3.6$, 1.2 Hz, 1 H), 7.46 (dd, $J = 5.1$, 1.1 Hz, 1 H), 7.14 (dd, $J = 5.1$, 3.6 Hz, 1 H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.6, 151.1, 150.4, 145.2, 144.3, 138.0, 132.0, 129.2, 126.9, 125.9, 124.2, 122.5; HRMS (ESI$^+$) m/z calcd for C$_{14}$H$_{10}$N$_5$O$_2$S (M+H)$^+$ 312.0550, found 312.0566.

4-(4-((6-Hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)phenyl)thiophene-2-carbonitrile (3.9j).

The requisite aniline was prepared by procedure 3.5 to yield 4-(4-aminophenyl)thiophene-2-carbonitrile which was carried forward crude as a beige solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 8.11 (d, $J = 1.5$ Hz, 1H), 7.89 (d, $J = 1.6$ Hz, 1H), 7.48 – 7.44 (m, 2H), 6.75 – 6.71 (m, 2H), 4.89 (br s, 2H).

Product 3.9j was synthesized using acetone as the solvent by procedure 3.1 (190 mg, 41%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 11.94 (br s, 1H), 9.63 (s, 1H), 8.31-8.29 (m, 1H), 8.26-8.21 (m, 3H), 7.89-7.84 (m, 2H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.6, 151.2, 150.4, 145.2, 143.1, 138.7, 137.5, 131.2, 128.5, 127.8, 122.6, 114.7, 111.1; HRMS (ESI$^-$) m/z calcd. for C$_{15}$H$_7$N$_6$O$_2$S (M-H) 335.0357, found 335.0367.
6-((3',5'-Difluoro-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9k).

The requisite aniline was prepared by procedure 3.5 to yield 3’,5’-difluoro-[1,1'-biphenyl]-3-amine in 85% as an orange oil: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 400 MHz) \(\delta\) 7.24 – 7.18 (m, 2H), 7.18 – 7.14 (m, 1H), 6.99 – 6.96 (m, 1H), 6.95 (dt, \(J = 9.1, 2.4\) Hz, 1H), 6.89 (ddd, \(J = 7.6, 1.8, 1.0\) Hz, 1H), 6.73 (ddd, \(J = 8.0, 2.3, 1.0\) Hz, 1H), 4.80 (br s, 2H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -111.48 – -111.55 (m, 2F).

Product 3.9k was synthesized using acetone as the solvent by procedure 3.1 (168 mg, 43%) as an off-white solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.09 (br s, 1H), 9.62 (s, 1H), 8.40 (d, \(J = 2.3\) Hz, 1H), 8.35-8.30 (m, 1H), 7.61-7.57 (m, 2H), 7.40-7.35 (m, 2H), 7.06 (tt, \(J = 9.1, 2.4\) Hz, 1H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -110.82 – -110.90 (m, 2F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 164.4 (dd, \(J_{CF} = 246.5, 13.5\) Hz), 153.6, 151.3, 150.4, 145.1, 145.0 (t, \(J_{CF} = 9.5\) Hz), 139.9 (t, \(J_{CF} = 2.7\) Hz), 139.4, 130.6, 124.3, 122.1, 120.7, 110.7 (dd, \(J_{CF} = 20.0, 6.3\) Hz), 103.5 (t, \(J_{CF} = 25.9\) Hz); HRMS (ESI) \(m/z\) calc'd. for C\(_{16}\)H\(_8\)F\(_2\)N\(_5\)O\(_2\) (M-H)\(^{-}\) 340.0652, found 340.0642.

6-((3’-(Trifluoromethoxy)-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9l).

Synthesized by procedure 3.3 to yield 3.9l (70 mg, 43%) as a yellow solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.09 (br s, 1H), 9.64 (s, 1H), 8.29-8.26 (m, 2H), 7.83-7.81 (m, 2H), 7.77-7.75 (m, 1H), 7.66-7.64 (m, 1H), 7.62 (t, \(J = 8.0\) Hz, 1H), 7.35-7.32 (m, 1H); \(^{19}\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -58.43 (s, 3F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 153.6, 151.2, 150.5 (q, \(J_{CF} = 1.9\) Hz), 150.4, 145.1, 143.4, 138.8, 136.5, 131.6, 128.3, 126.4, 122.6, 121.5 (q, \(J_{CF} = 255.5\) Hz), 120.5, 120.1; HRMS (ESI\(^{+}\)) \(m/z\) calc’d. for C\(_{17}\)H\(_{11}\)F\(_3\)N\(_5\)O\(_3\) (M+H)\(^{+}\) 390.0809, found 390.0811.

6-((3’-(Trifluoromethyl)-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9m).
Synthesized by procedure 3.3 to yield 3.9m (70 mg, 44%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.08 (br s, 1H), 9.65 (s, 1H), 8.30-8.27 (m, 2H), 8.02-8.01 (m, 2H), 7.87-7.84 (m, 2H), 7.73-7.71 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -63.09 (s, 3F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) $\delta$ 153.6, 151.2, 150.4, 145.1, 142.1, 138.8, 136.6, 131.6 (q, $J_{CF}$ = 31.9 Hz), 131.4, 130.8, 128.4, 125.4 (q, $J_{CF}$ = 271.7 Hz), 124.7 (q, $J_{CF}$ = 4.0 Hz), 124.1 (q, $J_{CF}$ = 3.9 Hz), 122.6; HRMS (ESI$^+$) m/z calc'd. for C$_{17}$H$_{11}$F$_3$N$_5$O$_2$ (M+H)$^+$ 374.0859, found 374.0859.

6-((3'-Methoxy-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9n).

Synthesized by procedure 3.1 to yield 3.9n (87mg, 25%) as a yellow solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 12.07 (s, 1H), 9.60 (s, 1H), 8.27 – 8.19 (m, 2H), 7.80 – 7.74 (m, 2H), 7.38 (t, 1H), 7.30 – 7.23 (m, 2H), 6.96 – 6.91 (m, 1H), 3.88 (s, 3H); $^{13}$C NMR (101 MHz, (CD$_3$)$_2$CO) $\delta$ 161.2, 153.6, 151.1, 150.5, 145.1, 142.5, 138.3, 138.1, 130.8, 128.2, 122.4, 119.8, 113.7, 113.1, 55.6; HRMS (ESI$^+$) calc’d for C$_{17}$H$_{14}$N$_5$O$_3$ (M+H)$^+$ 336.1097, found 336.1092.

6-((2,4'-Difluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9o).

The requisite aniline was prepared by procedure 3.5 to yield 2,4'-difluoro-[1,1'-biphenyl]-4-amine in 80% as a colorless solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) $\delta$ 7.52 – 7.47 (m, 2H), 7.20 – 7.13 (m, 3H), 6.57 (dd, $J$ = 8.3, 2.3 Hz, 1H), 6.50 (dd, $J$ = 13.5, 2.2 Hz, 1H), 5.08 (br s, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -118.18 – -118.27 (m, 1F), -119.93 (dd, $J$ = 13.4, 9.3 Hz, 1F).

Product 3.9o was synthesized using acetone as the solvent by procedure 3.1 (42 mg, 52%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.11 (br s, 1H), 9.73 (s, 1H), 8.24 (dd, $J$ = 13.3, 2.2 Hz, 1H), 8.01 (dd, $J$ = 8.5, 2.2 Hz, 1H), 7.68 – 7.64 (m, 2H), 7.60 (t, $J$ = 8.7 Hz, 1H), 7.29 – 7.24 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -116.05 – -116.27 (m, 1F), -119.93 (dd, $J$ = 13.4, 9.3 Hz, 1F).

Product 3.9o was synthesized using acetone as the solvent by procedure 3.1 (42 mg, 52%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ 12.11 (br s, 1H), 9.73 (s, 1H), 8.24 (dd, $J$ = 13.3, 2.2 Hz, 1H), 8.01 (dd, $J$ = 8.5, 2.2 Hz, 1H), 7.68 – 7.64 (m, 2H), 7.60 (t, $J$ = 8.7 Hz, 1H), 7.29 – 7.24 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -116.05 – -116.13 (m, 1F), -117.48 – -117.54 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) $\delta$ 163.3 (d, $J_{CF}$ = 245.6 Hz), 160.2 (d, $J_{CF}$ = 245.0 Hz), 153.4, 151.4, 150.2, 145.1, 139.6 (d, $J_{CF}$ = 11.3 Hz), 132.4 (d, $J_{CF}$ = 3.2 Hz), 131.7 (d, $J_{CF}$ = 3.2
Hz), 131.7 (d, $J_{CF} = 3.8$ Hz), 124.9 (d, $J_{CF} = 13.6$ Hz), 118.3 (d, $J_{CF} = 3.2$ Hz), 116.2 (d, $J_{CF} = 21.6$ Hz), 109.6 (d, $J_{CF} = 28.8$ Hz); HRMS (ESI) m/z calcd for $\text{C}_{16}\text{H}_{8}\text{F}_{2}\text{N}_{5}\text{O}_{2}$ (M-H$^-$) 340.0652, found 340.0644.

**6-((3,4'-Difluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-\text{b}]pyrazin-5-ol (3.9p).**

The requisite aniline was prepared by procedure 3.5 to yield 3,4'-difluoro-[1,1'-biphenyl]-4-amine in 74% as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 7.63 – 7.58 (m, 2H), 7.27 (dd, $J = 12.8, 2.1$ Hz, 1H), 7.21 (ddd, $J = 8.2, 2.1, 0.7$ Hz, 1H), 7.19 – 7.13 (m, 2H), 6.92 (dd, $J = 9.4, 8.2$ Hz, 1H), 4.81 (br s, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -118.62 – -118.70 (m, 1F), -136.99 (dd, $J = 12.8, 9.5$ Hz, 1F).

Product 3.9p was synthesized using acetone as the solvent by procedure 3.1 (209 mg, 11%) as a burnt-orange solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.17 (br s, 1H), 9.28 (s, 1H), 8.60 (t, $J = 8.5$ Hz, 1H), 7.82 – 7.76 (m, 2H), 7.65 – 7.61 (m, 2H), 7.29 – 7.24 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -116.22 – -116.27 (m, 1F), -127.72 – -128.78 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 163.7 (d, $J_{CF} = 245.6$ Hz), 155.2 (d, $J_{CF} = 245.8$ Hz), 153.6, 151.4, 150.3, 145.2, 139.0 (d, $J_{CF} = 7.6$ Hz), 136.2 (d, $J_{CF} = 2.7$ Hz), 129.7 (d, $J_{CF} = 8.3$ Hz), 125.5 (d, $J_{CF} = 10.6$ Hz), 124.3, 123.8 (d, $J_{CF} = 3.2$ Hz), 116.6 (d, $J_{CF} = 21.6$ Hz), 114.4 (d, $J_{CF} = 20.4$ Hz); HRMS (ESI) m/z calcd for $\text{C}_{16}\text{H}_{8}\text{F}_{2}\text{N}_{5}\text{O}_{2}$ (M-H$^-$) 340.0652, found 340.0654.

**6-((3'-((Difluoromethyl)-4'-fluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-\text{b}]pyrazin-5-ol (3.9q).**

Synthesized by procedure 3.3 to yield 3.9q (50 mg, 32%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.08 (br s, 1H), 9.63 (s, 1H), 8.28-8.25 (m, 2H), 7.96-7.92 (m, 2H), 7.82-7.79 (m, 2H), 7.43-7.39 (m, 2H), 7.43-7.39 (m, 1H), 7.16 (t, $J_{HF} = 54.6$ Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -114.52 – -114.68 (m, 2F), -122.90 – -122.98 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ
160.7 (dt, $J_{CF} = 251.1$, 5.1 Hz), 153.6, 151.2, 150.4, 145.1, 138.5, 138.0 (dt, $J_{CF} = 3.8$ Hz), 136.3, 132.1 (dt, $J_{CF} = 8.5$, 1.9 Hz), 128.2, 126.2 (dt, $J_{CF} = 6.2$, 3.0 Hz), 123.1 (dt, $J_{CF} = 23.1$, 12.8 Hz), 122.6, 117.6 (d, $J_{CF} = 20.8$ Hz), 112.8 (dt, $J_{CF} = 236.1$, 4.3 Hz).

HRMS (ESI$^+$) $m/z$ calc’d. for C$_{17}$H$_{11}$F$_3$N$_5$O$_2$ (M+H)$^+$ 374.0859, found 374.0860.

6-((4'-Fluoro-3'-hydroxy-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9r).

The requisite aniline was prepared by procedure 3.5 to yield 4'-amino-4-fluoro-[1,1'-biphenyl]-3-ol which was carried forward as a crude beige solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) $\delta$ 8.53 (s, 1H), 7.32 – 7.28 (m, 2H), 7.15 (dd, $J = 8.5$, 2.3 Hz, 1H), 7.08 (dd, $J = 11.0$, 8.5 Hz, 1H), 6.99 (ddd, $J = 8.4$, 4.3, 2.3 Hz, 1H), 6.74 – 6.70 (m, 2H), 4.73 (br s, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -143.01 – -143.08 (m, 1H).

Product 3.9r was synthesized by procedure 3.4 (74 mg, 27%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) $\delta$ O-H not visible 9.59 (s, 1H), 8.23 – 8.20 (m, 2H), 7.72 – 7.68 (m, 2H), 7.32 (dd, $J = 8.4$, 2.1 Hz, 1H), 7.22 – 7.15 (m, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) $\delta$ -140.75 – -140.82 (m, 1H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) $\delta$ 153.7, 152.1 (d, $J_{CF} = 240.9$ Hz), 151.2, 150.5, 146.0 (d, $J_{CF} = 13.2$ Hz), 145.2, 138.0, 137.5, 128.0, 122.4, 119.1 (d, $J_{CF} = 6.6$ Hz), 117.1 (d, $J_{CF} = 26.7$ Hz), 117.1, 117.0; HRMS (ESI$^+$) $m/z$ calcd for C$_{16}$H$_{11}$FN$_5$O$_3$ (M+H)$^+$ 340.0840, found 340.0833.

6-((4'-Fluoro-3'-methoxy-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9s).

The requisite aniline was prepared by procedure 5 to yield 4'-fluoro-3'-methoxy-[1,1'-biphenyl]-4-amine in 24% as a yellow oil: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) $\delta$ 7.37 – 7.35 (m, 2H), 7.27 – 7.24
(m, 1H), 7.14- 7.05 (m, 2H), 6.75 – 6.72 (m, 2H), 4.76 (br s, 2H), 3.95 (s, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -141.11 – -141.18 (m, 1F).

Product 3.9s was synthesized using acetone as the solvent by procedure 3.1 (119 mg, 60%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.06 (br s, 1H), 9.61 (s, 1H), 8.24 – 8.21 (m, 2H), 7.78-7.75 (m, 2H), 7.45 (dd, J = 8.3, 2.1 Hz, 1H), 7.28 – 7.20 (m, 2H), 4.01 (s, 3H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -138.81 – -138.87 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 153.6, 152.8 (d, $J_{CF}$ = 245.0 Hz), 151.2, 150.5, 148.9 (d, $J_{CF}$ = 10.8 Hz), 145.1, 138.1, 138.0 (d, $J_{CF}$ = 3.7 Hz), 137.7, 128.2, 122.5, 119.8 (d, $J_{CF}$ = 6.8 Hz), 116.9 (d, $J_{CF}$ = 18.5 Hz), 113.1 (d, $J_{CF}$ = 2.0 Hz), 56.6; HRMS (ESI$^+$) m/z calc’d. for C$_{17}$H$_{13}$FN$_5$O$_3$ (M+H)$^+$ 354.0997, found 354.1005.

4-Fluoro-4'-(6-hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)-[1,1'-biphenyl]-3-carbonitrile (3.9t).

The requisite aniline was prepared by procedure 3.5 to yield 4'-amino-4-fluoro-[1,1'-biphenyl]-3-carbonitrile in 90% as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 7.97 – 7.95 (m, 1H), 7.94 – 7.91 (m, 1H), 7.46 – 7.40 (m, 3H), 6.79 – 6.76 (m, 2H), 4.92 (br s, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -115.18 – -115.25 (m, 1F).

Product 3.9t was synthesized by procedure 3.1 (176 mg, 67%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$SO, 500 MHz) δ 13.32 (br s, 1H), 10.39 (s, 1H), 8.27 (dd, J = 6.1, 2.5 Hz, 1H), 8.19 – 8.16 (m, 2H), 8.13 – 8.09 (m, 1H), 7.81 – 7.77 (m, 2H), 7.61 (t, J = 9.0 Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -112.69 – -112.74 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$SO, 125 MHz) δ 161.8 (d, $J_{CF}$ = 255.9 Hz), 153.0, 150.8, 149.6, 144.6, 138.1, 137.0 (d, $J_{CF}$ = 3.3 Hz), 133.9 (d, $J_{CF}$ = 8.6 Hz) 133.1, 131.5, 127.1, 122.1, 117.1 (d, $J_{CF}$ = 19.5 Hz), 114.1, 100.8 (d, $J_{CF}$ = 15.4 Hz); HRMS (ESI$^+$) m/z calc’d. for C$_{17}$H$_8$FN$_6$O$_2$ (M-H)$^-$ 347.0698, found 347.0712.
3-Fluoro-4’-((6-hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)-[1,1'-biphenyl]-4-carbonitrile (3.9u).

The requisite aniline was prepared by procedure 3.5 to yield 4’-amino-3-fluoro-[1,1'-biphenyl]-4-carbonitrile in 84% as an off-white solid: ¹H NMR (400 MHz, (CD₃)₂SO) δ 13.43 (s, 1H), 10.43 (s, 1H), 8.24 – 8.20 (m, 2H), 8.00 (dd, J = 8.2, 7.1 Hz, 1H), 7.96 – 7.89 (m, 3H), 7.80 (dd, J = 8.2, 1.7 Hz, 1H); ¹⁹F NMR (376 MHz, (CD₃)₂SO) δ -108.17 (m, 1F); ¹³C NMR (126 MHz, (CD₃)₂SO) δ 163.2 (d, J₉CF = 253.4 Hz), 150.5, 148.3 (d, J₉CF = 8.7 Hz), 133.9, 128.1, 123.4 (d, J₉CF = 2.2 Hz), 121.5 (d, J₉CF = 2.4 Hz), 114.6, 114.0, 111.9 (d, J₉CF = 20.1 Hz), 95.9 (d, J₉CF = 15.4 Hz); HRMS (ESI⁺) calc’d for C₁₃H₁₀N₂ (M+H)⁺ 213.0823, found 213.0821.

Product 3.9u was synthesized by procedure 3.4 (240 mg, 96%) as a yellow solid: ¹H NMR (400 MHz, (CD₃)₂SO) δ 13.4 (s, 1H), 10.52 (s, 1H), 8.2 – 8.2 (m, 2H), 8.0 – 8.0 (m, 1H), 8.0 – 7.9 (m, 3H), 7.8 (dd, J = 8.2, 1.7 Hz, 1H); ¹⁹F NMR (376 MHz, (CD₃)₂SO) δ -108.1 – -108.2 (m, 1F); ¹³C NMR (126 MHz, (CD₃)₂SO) δ 163.0 (d, J₉CF = 254.4 Hz), 153.0, 150.9, 149.6, 147.0 (d, J₉CF = 9.2 Hz), 144.7, 139.1, 134.3, 132.7 (d, J₉CF = 2.9 Hz), 127.6, 123.2 (d, J₉CF = 3.5 Hz), 122.0, 114.3, 113.9 (d, J₉CF = 20.7 Hz), 98.3 (d, J₉CF = 15.5 Hz). HRMS (ESI⁺) calc’d for C₁₇H₈F₆N₆O₂ (M-H)⁻ 347.0698, found 347.0674.

2-((6-Hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)-9H-fluoren-9-one (9v).

Synthesized by procedure 3.1 at 75 °C to yield 3.9v (82 mg, 32%) as an orange solid: ¹H NMR ((CD₃)₂SO, 500 MHz) δ 13.34 (br s, 1H), 10.52 (s, 1H), 8.42 (d, J = 2.0 Hz, 1H), 8.20 (dd, J = 8.2, 2.1 Hz, 1H), 7.84 (d, J = 8.1 Hz, 1H), 7.76 (d, J = 7.6 Hz, 1H), 7.63 – 7.60 (m, 2H), 7.36 (t, J = 7.4 Hz, 1H); ¹³C NMR ((CD₃)₂SO, 125 MHz) δ 192.9, 152.9, 151.0, 149.5, 144.6, 143.9, 139.8, 139.1, 135.6, 133.9, 133.6, 129.1, 127.7, 124.1, 121.5, 121.0, 117.2; HRMS (ESI⁺) m/z calc’d. for C₁₇H₁₀N₅O₃ (M+H)⁺ 332.0778, found 332.0782.
6-((9,9-Dimethyl-9H-fluoren-2-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9w).

Synthesized by procedure 3.1 to yield 3.9w (160 mg, 49%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.07 (br s, 1H), 9.58 (s, 1H), 8.27 (d, $J$ = 2.1 Hz, 1H), 8.19 (dd, $J$ = 8.2, 2.1 Hz, 1H), 7.89 (d, $J$ = 8.3 Hz, 1H), 7.82 (dd, $J$ = 6.7, 1.4 Hz, 1H), 7.56-7.54 (m, 1H), 7.37-7.31 (m, 2H), 1.52 (s, 6H); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 155.3, 154.6, 153.7, 150.9, 150.5, 145.1, 139.5, 138.0, 137.0, 128.1, 128.0, 123.6, 121.2, 120.8, 116.7; HRMS (ESI$^-$) $m/z$ calc’d. for C$_{19}$H$_{14}$N$_5$O$_2$(M-H)$^-$ 344.1153, found 344.1122.

1-benzyl-5-(4-((6-hydroxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)amino)phenyl)piperidin-2-one (3.9x).

The requisite aniline was prepared by procedure 3.5 followed by Pd/C reduction to yield to 5-(4-aminophenyl)-1-benzylpiperidin-2-one as a off white solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) δ 7.37–7.22 (m, 5H), 6.96–6.90 (m, 2H), 6.69–6.61 (m, 2H), 4.67 (d, $J$ = 14.6 Hz, 1H), 4.53 (d, $J$ = 14.6 Hz, 1H), 3.24 (m, 1H), 2.95 (tt, $J$ = 10.4, 5.1 Hz, 1H), 2.56 (dd, $J$ = 7.9, 5.3 Hz, 2H), 2.03–1.95 (m, 2H). HRMS (ESI$^+$) calc’d for C$_{18}$H$_{21}$N$_2$O (M+H)$^+$ 281.1640, Found 281.1640.

Product 3.9x was synthesized by procedure 3.4 (291 mg, 34%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$SO, 400 MHz) δ 13.28 (s, 1H), 11.84 (s, 1H), 10.32 (s, 1H), 8.11–8.06 (m, 2H), 7.87 (dd, $J$ = 9.6, 2.8 Hz, 1H), 7.73 (d, $J$ = 2.7 Hz, 1H), 7.64–7.60 (m, 2H), 6.46–6.41 (m, 1H); $^{13}$C NMR ((CD$_3$)$_2$SO, 126 MHz) δ 162.1, 153.5, 151.1, 150.1, 144.9, 140.3, 136.9, 132.9, 132.8, 125.7, 122.5, 120.4, 117.9; HRMS (ESI$^-$) $m/z$ calc’d. for C$_{15}$H$_9$N$_6$O$_3$(M-H)$^-$ 321.0742, found 321.0730.

6-((1,1'-Biphenyl)-3-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9y).

Synthesized by procedure 3.2 to yield 3.9y (40 mg, 47%) as a yellow solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) δ 12.04 (s, 1H), 9.60 (s, 1H), 8.40 (t, $J$ = 1.8 Hz, 1H), 8.21 (dt, $J$ = 7.4, 1.9 Hz, 1H), 7.74–7.69 (m, 2H), 7.59–7.47 (m, 4H), 7.43–7.37 (m, 1H); $^{13}$C NMR (101 MHz, (CD$_3$)$_2$CO) δ
153.7, 151.4, 150.5, 145.2, 142.7, 141.4, 139.3, 130.4, 129.9, 128.6, 127.9, 124.4, 121.1, 120.7; HRMS (ESI+) calc’d for C_{22}H_{19}N_{6}O_{3} (M+-) 415.1524, found 415.1510.

6-((4'-Fluoro-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9z).

Synthesized by procedure 3.2 to yield 3.9z (9 mg, 23%) as a yellow solid: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)CO) \(\delta\) 9.60 (s, 1H), 8.37 (t, \(J = 2.0\), 1H), 8.24 – 8.18 (m, 1H), 7.80 – 7.71 (m, 2H), 7.60 – 7.48 (m, 2H), 7.27 (t, \(J = 9.0\), 2H); \(^1\)F NMR (376 MHz, (CD\(_3\))\(_2\)CO) \(\delta\) -116.8 – -116.9 (m, 1F); \(^{13}\)C NMR (126 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 162.0 (d, \(J_{CF} = 244.4\) Hz), 153.1, 150.9, 149.6, 144.6, 139.5, 138.4, 136.3 (d, \(J_{CF} = 3.1\) Hz), 129.3, 128.6 (d, \(J_{CF} = 8.2\) Hz), 123.0, 120.6, 120.1, 115.9 (d, \(J_{CF} = 21.5\) Hz); HRMS (ESI+) calc’d for C\(_{15}\)H\(_{13}\)FN\(_{5}\)O\(_{2}\) (M+H)\(^+\) 324.0891, found 324.0893.

6-((4'-Chloro-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9aa).

Synthesized by procedure 3.2 to yield 3.9aa (35 mg, 24%) as a yellow solid: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)CO) \(\delta\) 12.17 (s, 1H), 9.61 (s, 1H), 8.39 (t, \(J = 2.1\), 0.5 Hz, 3H), 8.28 – 8.19 (m, 1H), 7.79 – 7.69 (m, 2H), 7.64 – 7.46 (m, 2H); \(^{13}\)C NMR (101 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 153.7, 151.4, 150.9, 149.6, 144.6, 139.5, 141.3, 140.1, 139.4, 134.2, 130.5, 129.9, 129.5, 124.3, 121.4, 120.6; HRMS (ESI+) calc’d for C\(_{16}\)H\(_{11}\)ClN\(_{5}\)O\(_{2}\) (M+H)\(^+\) 340.0596, found 340.0595.

6-((4'-((Difluoromethoxy)-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ab).

Synthesized by procedure 3.3 to yield 3.9ab (61 mg, 43%) as an off-white solid: \(^1\)H NMR ((CD\(_3\))\(_2\)CO, 500 MHz) \(\delta\) 12.06 (br s, 1H), 9.61 (s, 1H), 8.40 (t, \(J = 2.0\), 1H), 8.23-8.20 (m, 1H), 7.79-7.76 (m, 2H), 7.56 (t, \(J = 7.8\), 1H), 7.52 (dt, \(J = 7.8\), 1.5 Hz, 1H), 7.33-7.30 (m, 2H), 7.07 (t, \(J_{HF} = 74.2\), 1H); \(^1\)F NMR ((CD\(_3\))\(_2\)CO, 376 MHz) \(\delta\) -82.74 (d, \(J_{HF} = 74.2\), 2F); \(^{13}\)C NMR ((CD\(_3\))\(_2\)CO, 125 MHz) \(\delta\) 153.7, 152.2 (t, \(J_{CF} = 3.1\) Hz), 151.3, 150.4, 145.1, 141.5, 139.3, 138.5,
130.4, 129.4, 124.3, 121.1, 120.6, 120.3, 117.5 (t, \( J_{CF} = 257.2 \) Hz); HRMS (ESI\(^+\)) \( m/z \) calc’d. for C\(_{17}\)H\(_{12}\)F\(_{2}\)N\(_{5}\)O\(_{3}\) (M+H\(^+\)) 372.0903, found 372.0915.

6-((4’-(Trifluoromethoxy)-[1,1’-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ac).

Synthesized by procedure 3.3 to yield 3.9ac (1.2 mg, 1%) as a yellow solid: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) 12.15 (s, 1H), 9.62 (s, 1H), 8.42 (t, \( J = 2.0 \) Hz, 1H), 8.25 (dt, \( J = 7.7, 1.8 \) Hz, 1H), 7.88 – 7.81 (m, 2H), 7.62 – 7.53 (m, 2H), 7.51 – 7.43 (m, 2H); \(^1\)H NMR (376 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) -58.6 (s, 3F); \(^{13}\)C NMR (126 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) 153.7, 151.4, 150.7, 150.4, 145.2, 144.6, 141.1, 140.6, 139.4, 130.5, 129.6, 124.5, 121.5, 121.2 (q, \( J = 293.8 \) Hz), 120.8; HRMS (ESI\(^+\)) calc’d for C\(_{17}\)H\(_{11}\)F\(_{3}\)N\(_{5}\)O\(_{3}\) (M+H\(^+\)) 390.0814, found 390.0826.

6-((4’-(Trifluoromethyl)-[1,1’-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ad).

Synthesized by procedure 3.2 to yield 3.9ad (57 mg, 55%) as a yellow solid: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) 12.0 (s, 1H), 9.7 (s, 1H), 8.5 (d, \( J = 1.9 \) Hz, 1H), 8.3 – 8.3 (m, 1H), 8.0 – 7.9 (m, 2H), 7.9 – 7.8 (m, 2H), 7.6 – 7.6 (m, 2H); \(^1\)F NMR (376 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) -62.9 (s, 3F), \(^{13}\)C NMR (101 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) 152.7, 150.5, 149.5, 144.3 (q, \( J_{CF} = 1.5 \) Hz), 144.2, 140.1, 138.5, 129.7, 129.0 (q, \( J_{CF} = 31.8 \) Hz), 127.6, 125.8 (q, \( J_{CF} = 4.0 \) Hz), 123.7, 121.9 (q, \( J_{CF} = 271.1 \) Hz), 121.0, 120.0; HRMS (ESI\(^+\)) calc’d for C\(_{17}\)H\(_{11}\)F\(_{3}\)N\(_{5}\)O\(_{2}\) (M+H\(^+\)) 374.0865, found 374.0861.

6-((3’-(Trifluoromethyl)-[1,1’-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ae).

Synthesized by procedure 3.2 to yield 3.9ae (8 mg, 4%) as a yellow solid: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) 11.68 (s, 1H), 9.67 (s, 1H), 8.47 – 8.42 (m, 1H), 8.36 – 8.28 (m, 1H), 8.07 – 7.99 (m, 2H), 7.79 – 7.72 (m, 2H), 7.65 – 7.59 (m, 2H); \(^1\)F NMR (376 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) -63.1 (s, 3F);
$^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) δ 153.6, 151.4, 150.4, 145.1, 142.4, 140.9, 139.5, 131.6 (q, $J_{CF}$ = 2.3 Hz), 131.6 (q, $J_{CF}$ = 32.5 Hz), 130.9, 130.6, 125.4 (q, $J_{CF}$ = 271.1 Hz), 125.2 (q, $J_{CF}$ = 4.3 Hz), 124.5, 124.3 (q, $J_{CF}$ = 4.3 Hz), 121.7, 120.9; HRMS (ESI$^+$) calc’d for C$_{17}$H$_1$F$_3$N$_5$O$_2$ (M+H)$^+$ 374.0865, found 374.0877.

6-((3',5'-Difluoro-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9af).

The requisite aniline was prepared by procedure 3.5 to yield 3',5'-difluoro-[1,1'-biphenyl]-3-amine in 85% as an orange oil: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 7.24 – 7.18 (m, 2H), 7.18 – 7.14 (m, 1H), 6.99 – 6.96 (m, 1H), 6.95 (dt, $J = 9.1$, 2.4 Hz, 1H), 6.89 (ddd, $J = 7.6$, 1.8, 1.0 Hz, 1H), 6.73 (ddd, $J = 8.0$, 2.3, 1.0 Hz, 1H), 4.80 (br s, 2H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -111.48 – -111.55 (m, 2F).

Product 3.9af was synthesized using acetone as the solvent by procedure 3.1 (218 mg, 43%) as an off-white solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.09 (br s, 1H), 9.62 (s, 1H), 8.40 (d, $J = 2.3$ Hz, 1H), 8.35–8.30 (m, 1H), 7.61-7.57 (m, 2H), 7.40-7.35 (m, 2H), 7.06 (tt, $J = 9.1$, 2.4 Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO, 376 MHz) δ -110.82 – -110.90 (m, 2F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 164.4 (dd, $J_{CF} = 246.5$, 13.5 Hz), 153.6, 151.3, 150.4, 145.1, 145.0 (t, $J_{CF} = 9.5$ Hz), 139.9 (t, $J_{CF} = 2.7$ Hz), 139.4, 130.6, 124.3, 122.1, 120.7, 110.7 (dd, $J_{CF} = 20.0$, 6.3 Hz), 103.5 (t, $J_{CF} = 25.9$ Hz); HRMS (ESI$^+$) m/z calc’d. for C$_{16}$H$_8$F$_2$N$_5$O$_2$ (M-H)$^-$ 340.0652, found 340.0642.

6-((3'-(Difluoromethyl)-4'-fluoro-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ag).

Synthesized by procedure 3.3 to yield 3.9ag (48 mg, 34%) as a yellow solid: $^1$H NMR ((CD$_3$)$_2$CO, 500 MHz) δ 12.09 (br s, 1H), 9.64 (s, 1H), 8.39-8.38 (m, 1H), 8.30-8.27 (m, 1H), 7.96-7.92 (m, 2H), 7.61-7.54 (m, 2H), 7.44 (t, $J = 9.3$ Hz, 1H), 7.18 (t, $J_{HF} = 54.6$ Hz, 1H); $^{19}$F NMR ((CD$_3$)$_2$CO,
376 MHz) δ -114.84 (dd, $J_{HF} = 54.6$ Hz, $J_{FF} = 4.0$ Hz, 2F), -122.43 – -122.51 (m, 1F); $^{13}$C NMR ((CD$_3$)$_2$CO, 125 MHz) δ 160.9 (dt, $J_{CF} = 251.6$, 5.3 Hz), 153.6, 151.3, 150.4, 145.1, 140.6, 139.4, 138.2 (d, $J_{CF} = 3.7$ Hz), 132.4 (dt, $J_{CF} = 8.6$, 2.0 Hz), 130.6, 126.4 (dt, $J_{CF} = 6.1$, 3.1 Hz), 124.3, 123.2 (dt, $J_{CF} = 23.2$, 12.9 Hz), 121.4, 120.7, 117.7 (d, $J_{CF} = 20.8$ Hz), 112.6 (dt, $J_{CF} = 236.3$, 4.5 Hz); HRMS (ESI$^+$) $m/z$ calc’d for C$_{17}$H$_{11}$F$_3$N$_5$O$_2$ (M+H)$^+$ 374.0859, found 374.0855.

6-((4'-Methyl-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ah).

Synthesized by procedure 3.2 to yield 3.9ah (57 mg, 64%) as a yellow solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) δ 11.4 (s, 1H), 9.6 (s, 1H), 8.4 (t, $J = 1.8$ Hz, 1H), 8.2 (dt, $J = 7.5$, 2.0 Hz, 1H), 7.6 – 7.7 (m, 2H), 7.5 – 7.5 (m, 2H), 7.3 – 7.3 (m, 2H), 2.4 (s, 3H); $^{13}$C NMR (101 MHz, (CD$_3$)$_2$CO) δ *rotamers*153.6, 153.5*, 151.2, 151.1*, 150.4, 145.0, 142.5, 139.1, 139.0*, 138.4, 138.2, 130.4, 130.2*, 127.6, 124.1, 124.0, 120.6, 120.5, 120.4, 120.3, 21.1; HRMS (ESI$^+$) calc’d for C$_{17}$H$_{14}$N$_5$O$_2$ (M+H)$^+$ 320.1142, found 320.1137.

6-((3-(Benzo[d][1,3]dioxol-5-yl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ai).

The requisite aniline was prepared by procedure 3.5 to yield 3-(benzo[d][1,3]dioxol-5-yl)aniline in 62% as a yellow oil: $^1$H NMR ((CD$_3$)$_2$CO, 400 MHz) δ 7.14 – 7.05 (m, 3H), 6.90 – 6.87 (m, 2H), 6.79 (ddd, $J = 7.6$, 1.8, 1.0 Hz, 1H), 6.62 (ddd, $J = 8.0$, 2.3, 1.0 Hz, 1H), 6.02 (s, 2H), 4.67 (br s, 2H).

Product 3.9ai was synthesized using acetone as the solvent by procedure 3.1 (212 mg, 54%) as a brown solid: $^1$H NMR ((CD$_3$)$_2$SO, 500 MHz) δ 13.31 (br s, 1H), 10.27 (s, 1H), 8.24 (t, $J = 2.0$ Hz, 1H), 8.06 (dt, $J = 8.1$, 1.6 Hz, 1H), 7.48 – 7.41 (m, 2H), 7.23 (d, $J = 1.8$ Hz, 1H), 7.16 (dd, $J = 8.1$, 1.8 Hz, 1H), 7.04 (d, $J = 8.0$ Hz, 1H), 6.08 (s, 2H); $^{13}$C NMR ((CD$_3$)$_2$SO, 125 MHz) δ 153.1, 150.9,
149.7, 148.1, 147.1, 144.6, 140.3, 138.3, 134.1, 129.2, 122.8, 120.22, 120.19, 119.9, 108.8, 107.0, 101.2; HRMS (ESI) \( m/z \) calc'd. for C\(_{17}\)H\(_{10}\)N\(_5\)O\(_4\) (M-H)\(^-\) 348.0738, found 348.0735.

6-((3-(2,2-Difluorobenzo[d][1,3]dioxol-5-yl)phenyl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9aj).

The requisite aniline was prepared by procedure 3.5 to yield 3-(2,2-difluorobenzo[d][1,3]-dioxol-5-yl)aniline in 95% as an oil: \(^1\)H NMR ((CD\(_3\)\(_2\)CO, 400 MHz) \( \delta \) 7.47 (dd, \( J = 1.8, 0.6 \) Hz, 1H), 7.40 (ddd, \( J = 8.3, 1.8, 0.6 \) Hz, 1H), 7.32 (dt, \( J = 8.5, 0.6 \) Hz, 1H), 7.14 (t, \( J = 7.8 \) Hz, 1H), 6.94 – 6.92 (m, 1H), 6.84 (ddd, \( J = 7.7, 1.9, 1.0 \) Hz, 1H), 6.69 (ddd, \( J = 8.0, 2.3, 1.0 \) Hz, 1H), 4.77 (br s, 2H); \(^{19}\)F NMR ((CD\(_3\)\(_2\)CO, 376 MHz) \( \delta \) -51.20 (d, \( J = 1.8 \) Hz, 2F).

Product 3.9aj was synthesized using acetone as the solvent by procedure 3.1 (169 mg, 45%) as an off-white solid: \(^1\)H NMR ((CD\(_3\)\(_2\)CO, 500 MHz) \( \delta \) 12.08 (br s, 1H), 9.60 (s, 1H), 8.36 (t, \( J = 2.0 \) Hz, 1H), 8.26 – 8.24 (m, 1H), 7.62 (d, \( J = 1.8 \) Hz, 1H), 7.58 – 7.51 (m, 3H), 7.41 (d, \( J = 8.3 \) Hz, 1H); \(^{19}\)F NMR ((CD\(_3\)\(_2\)CO, 376 MHz) \( \delta \) -51.14 (s, 2F); \(^{13}\)C NMR ((CD\(_3\)\(_2\)CO, 125 MHz) \( \delta \) 153.6, 151.3, 150.4, 145.1, 145.0, 144.0, 141.2, 139.3, 138.4, 132.6 (t, \( J_{CF} = 252.7 \) Hz), 130.5, 124.4, 123.9, 121.3, 120.7, 111.0, 109.4; HRMS (ESI) \( m/z \) calc’d. for C\(_{17}\)H\(_8\)F\(_2\)N\(_5\)O\(_4\) (M-H)\(^-\) 384.0550, found 384.0554.

6-((5-(Trifluoromethoxy)-4'-(trifluoromethyl)-[1,1'-biphenyl]-2-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol (3.9ak).

The requisite aniline was prepared by procedure 3.5 to yield 5-(trifluoromethoxy)-4'-(trifluoromethyl)-[1,1'-biphenyl]-2-amine in 48% as an off-white solid: \(^1\)H NMR (400 MHz, Acetone-\(d_6\)) \( \delta \) 7.83 – 7.79 (m, 2H), 7.74 – 7.70 (m, 2H), 7.13 – 7.08 (m, 1H), 7.06 – 7.03 (m, 1H), 7.00 – 6.94 (m, 2H), 6.81 (ddd, \( J = 7.7, 2.0, 1.0 \) Hz, 1H), 4.78 (br s, 2H); \(^{19}\)F NMR ((CD\(_3\)\(_2\)CO, 376 MHz) \( \delta \) -51.12 (s, 2F).
6.93 (dd, $J = 8.7$, 0.5 Hz, 1H), 4.83 (s, 2H); $^{19}$F NMR (376 MHz, (CD$_3$)$_2$CO) $\delta$ -59.2 (s, 3F), -63.0 (s, 3F); HRMS (ESI$^+$) calc’d for C$_{14}$H$_{10}$F$_6$NO (M+H)$^+$ 322.0661, found 322.0659.

Product 3.9ak was synthesized by procedure 3.4 (128 mg, 50%) as a yellow solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 12.07 (s, 1H), 9.23 (s, 1H), 8.54 (d, $J = 9.0$ Hz, 1H), 7.89 – 7.77 (m, 4H), 7.60 – 7.55 (m, 1H), 7.49 – 7.45 (m, 1H); $^{19}$F NMR (376 MHz, (CD$_3$)$_2$CO) $\delta$ -58.5 – -58.6 (m, 3F), -63.0 – -63.1 (m, 3F); $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) $\delta$ 153.6, 152.1, 150.4, 147.3 (q, $J_{CF} = 2.1$ Hz), 145.2, 141.8 (q, $J_{CF} = 2.7$ Hz), 137.0, 134.3, 131.1, 130.9 (q, $J_{CF} = 32.3$ Hz), 126.9, 126.9 (q, $J_{CF} = 4.0$ Hz), 125.3 (q, $J_{CF} = 270.9$ Hz), 124.0, 122.5, 121.6 (q, $J_{CF} = 256.5$ Hz); HRMS (ESI$^+$) calc’d for C$_{18}$H$_{10}$F$_6$N$_5$O$_2$ (M+H)$^+$ 458.0682, found 458.0684.

N-(4'-fluoro-[1,1'-biphenyl]-4-yl)-6-methoxy-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-amine (3.10).

Synthesized by procedure 3.1 in acetone to yield 3.10 (118 mg, 65%) as a burnt-orange solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 9.64 (s, 1H), 8.21 – 8.10 (m, 2H), 7.79 – 7.70 (m, 4H), 7.30 – 7.19 (m, 2H), 4.24 (s, 3H); $^{19}$F NMR (376 MHz, (CD$_3$)$_2$CO) $\delta$ -117.27 – -117.35 (m); $^{13}$C NMR (126 MHz, Acetone-$d_6$) $\delta$ 163.3 (d, $J = 244.7$ Hz), 156.5, 151.9, 150.6, 147.6, 138.1, 137.5 (d, $J = 3.2$ Hz), 137.4, 129.4 (d, $J = 8.1$ Hz), 128.0, 122.9, 116.5 (d, $J = 21.7$ Hz), 56.5; HRMS (ESI$^+$) m/z calcd for C$_{16}$H$_{10}$N$_5$O$_2$ (M-H)$^-$ 337.1048, found 338.1068.
4.3.6 NMR Spectra for compounds tested In Vivo.

6-((1,1′-Biphenyl)-4-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) (3.9a).

6-((1,1′-Biphenyl)-4-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) (3.9a).
6-((4'-Fluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol \textsuperscript{1}H NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}CO) (3.9b).

6-([1,1'-Biphenyl]-4-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol LCMS (3.9a).

RT: \textit{0.000 - 21.953}
6-((4'-Fluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{19}$F NMR (400 MHz, (CD$_3$)$_2$CO) (3.9b).

6-((4'-Fluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) (3.9b).
6-((4'-Fluoro-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol LCMS (3.9b).

6-((3'-(Trifluoromethyl)-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) (3.9m).
6-((3′-(Trifluoromethyl)-[1,1′-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{19}$F NMR (400 MHz, (CD$_3$)$_2$CO) (3.9m).

6-((3′-(Trifluoromethyl)-[1,1′-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) (3.9m).
6-((3’-(Trifluoromethyl)-1,1’-biphenyl)-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol
LCMS (3.9m).

6-((3’-Methoxy-[1,1'-biphenyl]-4-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol
$^1$H NMR (400 MHz, (CD$_3$)$_2$CO) (3.9n).
6-((3'-Methoxy-[1,1'-biphenyl]-4-yl)amino)[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) (3.9n)

6-((3'-Methoxy-[1,1'-biphenyl]-4-yl)amino)[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol LCMS (3.9n)
6-([1',1'-Biphenyl]-3-ylamino)-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) (3.9y).

6-([1',1'-Biphenyl]-3-ylamino)-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) (3.9y).
6-((1,1'-Biphenyl)-3-ylamino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol LCMS (3.9y).

6-((4'-(Trifluoromethyl)-1,1'-biphenyl)-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol \(^1\)H NMR (400 MHz, \(\text{CD}_3\text{CO}) \) (3.9ad).
6-((4')(Trifluoromethyl)-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{19}$F NMR (400 MHz, (CD$_3$)$_2$CO) (3.9ad).

6-((4'(Trifluoromethyl)-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) (3.9ad).
6-((4'-(Trifluoromethyl)-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol LCMS (3.9ad).

6-((4'-Methyl-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-b]pyrazin-5-ol \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)CO) (3.9ah).
6-((4'-Methyl-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol $^{13}$C NMR (126 MHz, (CD$_3$)$_2$CO) (3.9ah).

6-((4'-Methyl-[1,1'-biphenyl]-3-yl)amino)-[1,2,5]oxadiazolo[3,4-\textit{b}]pyrazin-5-ol LCMS (3.9ah).