Discovery and synthetic optimization of a novel scaffold for hydrophobic tunnel-targeted autotaxin inhibition

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Abstract

Autotaxin (ATX) is a ubiquitous ectoenzyme that hydrolyzes lysophosphatidylcholine (LPC) to form the bioactive lipid mediator lysophosphatidic acid (LPA). LPA activates specific G-protein coupled receptors to elicit downstream effects leading to cellular motility, survival, and invasion. Through these pathways, upregulation of ATX is linked to diseases such as cancer and cardiovascular disease. Recent crystal structures confirm that the catalytic domain of ATX contains multiple binding regions including a polar active site, hydrophobic tunnel, and a hydrophobic pocket. This finding is consistent with the promiscuous nature of ATX hydrolysis of multiple and diverse substrates and prior investigations of inhibitor impacts on ATX enzyme kinetics. The current study used virtual screening methods to guide experimental identification and characterization of inhibitors targeting the hydrophobic region of ATX. An initially discovered inhibitor, GRI392104 (IC₅₀ 4 μ M) was used as a lead for synthetic optimization. In total twelve newly synthesized inhibitors of ATX were more potent than GRI392104 and were selective for ATX as they had no effect on other LPC-specific NPP family members or on LPA₁₋₅ GPCR.

Keywords: autotaxin; structure-activity relationship; pharmacophore

Abbreviations

ATX/NPP2 – Autotaxin
DIPEA – *N,N*-diisopropylethylamine
DMF – *N,N*-dimethylformamide
DMSO – dimethylsulfoxide
GPCR – G-protein coupled receptor
GRI – Genomic Research Institute
HBTU – *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
LPC – lysophosphatidylcholine
LPA – lysophosphatidic acid
LysoPLD – lysophospholipase-D
MOE – Molecular Operating Environment
NPP – nucleotide pyrophosphatase phosphodiesterase
pNP-TMP – *p*-nitrophenyl thymidine-5'-monophosphate
pNPPC – *para*-nitrophenylphosphocholine

1. Introduction

Autotaxin (ATX, NPP2, lysoPLD) is a ubiquitous, secreted enzyme in the nucleotide pyrophosphatase/phosphodiesterase (NPP) family.[1-4] NPP1-4 are capable of hydrolyzing phosphoanhydrides whereas NPP 2, 6, and 7 hydrolyze phosphodiester linkages.[1,5,6] To date the natural substrate for NPP5 remains unknown. Within this family only ATX is capable of cleaving both phosphoanhydride and phosphate ester bonds, although it exhibits preference for phosphate esters.[2,3,7,8] Regiochemically, ATX has lysophospholipase-D (lysoPLD) activity, allowing it to hydrolyze lysophosphatidylcholine (LPC) to generate the bioactive lipid, lysophosphatidic acid (LPA) and choline (scheme 1).[1-3] Through this lysoPLD activity, ATX is the primary source of plasma LPA.[9] Much of the biological activity of ATX can be attributed to LPA, which prompts several signaling cascades through the activation of specific G-protein coupled receptors which stimulate cell proliferation, survival and migration.[10-12] Through this downstream signaling of LPA, ATX is necessary for embryonic development of the neural tube and also plays a role in wound healing.[13-15] ATX also plays an essential role in blood vessel formation during embryogenesis as knockouts are non-viable due to defects in angiogenesis.[16] However, upregulated ATX and subsequently increased level of LPA have been linked to oncogenic transformation, cancers metastasis and therapeutic resistance, cardiovascular disease, Alzheimer's disease, and neuropathic pain.[3,11,17-19] The relationship between ATX and human disease makes it a potential therapeutic target. The goal of this project is to discover novel small-molecule non-lipid drug-like inhibitors of ATX by use of a structure-based pharmacophore, targeting the hydrophobic tunnel of ATX.

Scheme 1: ATX-catalyzed hydrolysis of lysophosphatidylcholine to lysophosphatidic acid.

Pharmacophores are geometrical models of structural features important for biological activity.[20] Pharmacophores can be either ligand-based, where ligand commonalities alone are utilized, or structure-based, where ligand similarities are taken into account in context of their interactions in a target protein.[21] North *et al.*, Mize *et al.*, and Norman *et al.* utilized ligand-based techniques to develop pharmacophores for ATX, but the present work differs because it is one of the first structure-based pharmacophores for ATX (Fells *et al.* also reported a structure-based pharmacophore targeting the hydrophobic pocket of ATX).[22-25] Recent crystallized structures of ATX are useful tools from which to develop structure-based pharmacophores.[26-30] Crystal structures of mouse, rat, and human ATX all are composed of three main domains, including a catalytic domain, which contains a polar active site, a hydrophobic tunnel, and a hydrophobic pocket (figure 1). The prevalence of non-polar amino acid sidechains in the hydrophobic tunnel of ATX might lead to a structure-based pharmacophore that contains predominantly non-specific hydrophobic features, capable of finding compounds which fit into the ATX hydrophobic pocket but may also bind to other receptors. Aromatic features can provide essential interaction directionality that can potentially improve specificity as aromatic rings show strong preference to interact in either an edge-to-face or face-to-face orientation.[31] Aromatic features were deliberately included in the pharmacophore utilized here in high-throughput virtual screening of large databases to discover a variety of new and hopefully selective scaffolds for potential inhibitors which may be worthwhile to pursue further with structure-activity relationship studies.

Figure 1: One of the recently published ATX mouse crystal structures (PDB ID: 3NKM).[27,33] The two N-terminal somatomedin-B-like domains are in blue. The catalytic domain is pink and shows the surface around the binding sites; hydrophobic surfaces are green and polar surfaces are purple. The two zinc ions in the active site are shown in teal. The nuclease-like domain is dark red. The two linker regions are dark gray.

In this paper, we describe the formation of a structure-based pharmacophore which lead to the discovery of several hydrophobic, yet non-lipid inhibitors of ATX. These compounds docked within the same volume occupied by the initial non-lipid inhibitors of ATX used to build the pharmacophore. Violations to Lipinski's Rule of Five were calculated for each compound to filter out compounds that are not drug-like.[32] In order to sample the entirety of the chemical space found by the pharmacophore, compounds were grouped together into clusters based on similarity. Representatives from each cluster were tested for ATX inhibition using two assays, one using a FRET-based substrate, FS-3, and the other using a nucleotide substrate, *p*-nitrophenyl 5'-thymidine monophosphate. Of the seventy-two compounds tested, four inhibited FS-3 hydrolysis of ATX by 50% or greater at a concentration of 10 μ M. Sixty-six analogs of one lead were synthesized to explore the structure-activity relationship of this novel scaffold. Thirty-six compounds inhibited ATX-catalyzed FS-3 hydrolysis by 50% or greater at a concentration of 10 μ M, with one compound having a sub-micromolar potency.

2. Experimental Methods

2.1. ATX Protein Model Development

To compare known ATX structures and to prepare a useful template for structure-based pharmacophore development for AXT inhibition, crystal structures of mouse (PDB ID: 3NKM[27] and rat (PDB ID: 2XR9[26]) ATX were downloaded from the Protein Data Bank (PDB[33]) and the FASTA sequence of human ATX (Q13822) was obtained from the National Center for Biotechnology Information.[34] Since the human ATX sequences show high sequence similarity to the mouse (95%) and rat (94%) homologues, the available mouse and rat structures were carefully scrutinized using MOE (Molecular Operating Environment, Chemical Computing Group, Montreal, Canada). The rat and mouse tertiary structures are very similar (0.70 Å alpha carbon RMSD) as would be expected based on their similar primary sequences. However, the rat structure lacked several backbone atoms. Likewise, the mouse structure contained fifteen residues truncated to alanine. These truncations were spread across three regions: the N-terminal domain (K59, E67, and K104), the catalytic domain (R162, R244, F274, N398, L458, and K462), and the nuclease-like domain (R549, Q559, R602, E642, and K666). Full amino acids sidechains (appropriate for each position) were added to the mouse structure MOE using the structure preparation tool, the corrected structure was energy minimized and validated through aligned with crystal structure (PDB ID: 3WAV[35]).

2.2. Pharmacophore Creation

Two potent ATX inhibitors, KM04131[22] and PF8380[36] were previously docked into mouse ATX.[22] A consensus pharmacophore was built where similar annotation points from these structurally distinct ligands would become a feature point for the model. The annotation points were predominately hydrophobic or aromatic, leading to a pharmacophore with hydrophobic and aromatic features. An exclusion volume shape with a radius of 4Å, was added to the pharmacophore to make use of the structural information obtained from docking. The exclusion volume shape excludes compounds which dock outside of the general volume of the initial lead compounds. This pharmacophore was analyzed against an internal database of 457 compounds with measured ATX inhibitory activity (111 actives and 346 inactives)[22] to calculate performance metrics (equations 1-5).[37] The resulting pharmacophore was used to search the Genomic Research Institute (GRI) database of ~340,000 searchable compounds with unknown ATX activity from the University of Cincinnati Drug Discovery Center (UC-DDC).

2.3. *In Silico* Candidate Inhibitor Screening

Candidate compounds, selected using the structure- based pharmacophore, were docked into the crystal structure of mouse ATX to observed modeled interactions. The receptor was prepared with Autodock Tools for use as a docking target by Autodock Vina.[38] A grid box was also prepared in Autodock Tools, to focus the docking search into the catalytic domain of ATX. The box center had x,y,z coordinates of 21.383, 36.532, and 7.403 (within 3 Å of the backbone carbonyl oxygens of both Lys 208 and Asp 358 as well as the sidechain amide of Asn 212 and the β carbon of His 359) respectively, with x,y,z side lengths of 40 Å, 30 Å, and 30 Å. All GRI compounds were docked flexibly into the rigid ATX structure using Autodock Vina.

Structures selected by the pharmacophore that docked within the exclusion volume shape were analyzed with Lipinski's rules to exclude those exhibiting structural features not commonly found in orally bioavailable drugs.[32] Those with greater than 5 hydrogen-bond donors, 10 hydrogen-bond acceptors, a log partition coefficient above 5, or a molecular weight above 500 Daltons were removed from the candidate set. The remaining candidates were clustered into groups by similarity using the Tanimoto coefficient[39] calculated on the basis of MACCS structural keys (MDL Information Systems Inc., San Leandro, California). Those within 55% similarity of one another were grouped into clusters. In order to sample a variety of chemical scaffolds, visual inspection of the most representative scaffold in each cluster lead to selecting 72 unique compounds for *in vitro* screening.

2.4. Enzyme Expression and Purification

Human FLAG-fusion constructs of ATX, NPP6, and NPP7 were expressed and purified as previously described.[40]

2.5. *In Vitro* Screening

Two ATX activity assays were used as previously described.[24] The primary ATX activity assay used FS-3 (Echelon Biosciences, Salt Lake City, Utah[41]), a long substrate which contains a dabcyl quencher. When hydrolyzed by ATX, the carboxyfluorescein fluorophore, becomes detectable by fluorescence. A secondary ATX activity assay used *para*-nitrophenyl thymidine-5'-monophosphate (pNP-TMP, Sigma-Aldrich, St. Louis, Missouri), a short substrate which releases a detectable chromophore, *para*-nitrophenolate. All assays were performed in triplicate using 96-well half-area plates (Corning, Corning, New York) and were monitored using a BioTek Synergy 2 multi-detection microplate reader (BioTek Instruments, Winooski, Vermont). Control assays were done using 10 μ M inhibitor candidate with and without 100 nM carboxyfluorescein (Sigma-Aldrich) or 100 μ M *para*-nitrophenol (Sigma-Aldrich), as assay controls. A preliminary single dose assay to determine ATX inhibition consisted of 10 μ M compound, 10 nM ATX in assay buffer (30 μ M BSA in 50 mM Tris pH 8.0, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM KCl, and 140 mM NaCl), and either 1 μ M FS-3 or 1 mM pNP-TMP. For the carboxyfluorescein control and FS-3 assays, fluorescence was recorded at excitation and emission wavelengths (485 and 520 nm, respectively) for 60 minutes at 37°C. Absorbance was recorded at 405 nm for the same length of time and temperature for the *para*-nitrophenolate and pNP-TMP assays. Readings were taken every two minutes and normalized between a negative control (substrate with no ATX, set to 0%) and a positive control (substrate with ATX and no compound, set to 100%). A dose response assay was done for compounds showing greater than 50% inhibition at 10 μ M, with inhibitor concentrations ranging from 0.03 μ M to 30 μ M, 10 nM ATX in assay buffer, and either 1 μ M FS-3 or 1 mM pNP-TMP. For dose response results, non-linear regression analysis was done to determine the IC_{50} for each inhibitor (GraphPad Software Inc., La Jolla, California).

2.6. ATX Inhibitor Specificity

Specificity for ATX inhibition was determined by measuring NPP6/7 induced hydrolysis of a synthetic substrate, *para*-nitrophenylphosphocholine (pNPPC, Sigma-Aldrich, St. Louis, Missouri) which releases *para*-nitrophenolate. Assays were performed in triplicate using 96-well half-area plates (Corning, Corning, New York) with absorbance monitored at 405 nm and 37 °C using a BioTek Synergy 2 multi-detection microplate reader (BioTek Instruments, Winooski, Vermont). Final concentrations on the plate were 10 µM ATX inhibitor, 10 nM NPP6 in assay buffer (500 mM NaCl, 100 mM Tris-HCl, 0.05% Triton X-100, pH 9) or 10 nM NPP7 in assay buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM taurocholate, pH 8), and 10 µM pNPPC in appropriate assay buffer. Readings were taken every two minutes for an hour and normalized between a negative control (no enzyme, set to 0%) and a positive control (with enzyme, set to 100%). After background absorbance was subtracted for each compound, data was reported as a mean percent response relative to the positive control.

2.7. ATX Inhibitor Specificity: LPA₁₋₅ Assays

To confirm ATX specificity of **22**, calcium mobilization assays were completed for LPA₁₋₅ as previously described using a FlexStation plate reader (Molecular Devices, San Diego, California).[42]

2.8. GRI 392104 Analog Design

A structure-activity relationship examination was undertaken for the most potent and synthetically tractable inhibitor (GRI 392104) identified from this initial screen. Changes in the lead included shortening the flexible carbon chain (linker), changing the position of the nitro group on the phenyl piperazine segment, and altering the halogen substituents on the terminal ring (table 1).

GRI 392104

GRI 392104 Analog Scaffold

Table 1: GRI 392104 analog design

Analog	Y ₁	Y ₂	Y ₃	X ₁	X ₂	X ₃	X ₄
1,2,3	NO ₂	---	---	Cl, F, CF ₃	---	---	---
4,5,6	NO ₂	---	---	---	Cl, F, CF ₃	---	---
7,8,9	NO ₂	---	---	---	---	Cl, F, CF ₃	---
10	NO ₂	---	---	Cl	---	Cl	---
11	NO ₂	---	---	---	Cl	---	Cl
12,13,14	---	NO ₂	---	Cl, F, CF ₃	---	---	---
15,16,17	---	NO ₂	---	---	Cl, F, CF ₃	---	---
18,19,20	---	NO ₂	---	---	---	Cl, F, CF ₃	---
21	---	NO ₂	---	Cl	---	Cl	---
22	---	NO ₂	---	---	Cl	---	Cl
23,24,25	---	---	NO ₂	Cl, F, CF ₃	---	---	---
26,27,28	---	---	NO ₂	---	Cl, F, CF ₃	---	---
29,30,31	---	---	NO ₂	---	---	Cl, F, CF ₃	---
32	---	---	NO ₂	Cl	---	Cl	---
33	---	---	NO ₂	---	Cl	---	Cl
34,35,36	COOH	---	---	Cl, F, CF ₃	---	---	---
37,38,39	COOH	---	---	---	Cl, F, CF ₃	---	---
40,41,42	COOH	---	---	---	---	Cl, F, CF ₃	---
43	COOH	---	---	Cl	---	Cl	---
44	COOH	---	---	---	Cl	---	Cl
45,46,47	---	COOH	---	Cl, F, CF ₃	---	---	---
48,49,50	---	COOH	---	---	Cl, F, CF ₃	---	---
51,52,53	---	COOH	---	---	---	Cl, F, CF ₃	---
54	---	COOH	---	Cl	---	Cl	---
55	---	COOH	---	---	Cl	---	Cl
56,57,58	---	---	COOH	Cl, F, CF ₃	---	---	---
59,60,61	---	---	COOH	---	Cl, F, CF ₃	---	---
62,63,64	---	---	COOH	---	---	Cl, F, CF ₃	---
65	---	---	COOH	Cl	---	Cl	---
66	---	---	COOH	---	Cl	---	Cl

Scheme 2: Synthetic scheme to make analogs of GRI 392104 with $R=NO_2$ or $COOH$ and $X=F$, Cl (mono), Cl (di), CF_3 . Activation of the carboxylic acid requires O-(Benzotriazol-1-yl)-N,N,N',N',-tetramethyluronium hexafluorophosphate (HBTU) with diisopropylethylamine (DIPEA).

2.8.1. General Procedure for Nitro Analog Synthesis

Thirty-three nitro-containing analogs of GRI392104 (table 1) were synthesized using a two-step reaction (scheme 2). To a dry round bottom flask, 190 μ mol of carboxylic acid building block, 190 μ mol of HBTU (O-(benzotriazol-1-yl)-N, N, N',N'-tetramethyluronium hexafluorophosphate) and 190 μ mol of N,N-diisopropylethylamine (DIPEA) were combined in 1 mL of N,N-dimethylformamide (DMF) and stirred for 5 minutes at room temperature. After the solid carboxylic acid had dissolved and the clear solution turned amber, 97 μ mol of amine building block was dissolved in 1 mL of DMF and added to the reaction flask. All reactions were complete within 20 minutes, as confirmed by analytical thin layer chromatography (TLC) in 60:40 ethyl acetate/hexanes. The resulting amide products were extracted into chloroform and washed with water (twice), sodium bicarbonate (once), and water again (three times). The organic layers were dried and 1H -NMR (in $CDCl_3$) was used to ensure product formation. Individual products (as yellow solids) were isolated using preparative TLC with a mobile phase of 60:40 ethyl acetate/hexanes followed by extraction from scraped silica using 50:50 ethyl acetate/methylene chloride. Purity for all thirty-three compounds was confirmed by 1H -NMR, ^{13}C -NMR and LC-MS. High resolution MS data for all previously unreported compounds gave the expected mass values with absolute errors ranging from 0.5 to 13.9 ppm.

(1) 2-(2-chlorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 17.9 mg of yellow solid (49%). R_f = 0.56 (ethyl acetate/hexanes 60:40). 1H -NMR (chloroform- d): δ = 3.03 (m, 4H), 3.79 (m, 4H), 4.79 (s, 2H), 6.94 (t, 1H), 7.01 (d, 1H), 7.11 (t, 2H), 7.20 (d, 1H), 7.36 (d, 1H), 7.49 (t, 1H), 7.78 (d, 1H). MS (ESI+): m/z = 376.21 $[M+H]^+$, 378.22 $[M+2+H]^+$.

(2) 2-(2-fluorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 13.3 mg of yellow solid (35%). R_f = 0.63 (ethyl acetate/hexanes 60:40). 1H -NMR (chloroform- d): δ = 3.05 (t, 4H), 3.78 (t, 4H), 4.78 (s, 2H), 6.95 (m, 1H), 7.08 (m, 5H), 7.50 (t, 1H), 7.78 (d, 1H). MS (ESI+): m/z = 360.23 $[M+H]^+$.

(3) 1-(4-(2-nitrophenyl)piperazin-1-yl)-2-(2-(trifluoromethyl)phenoxy)ethanone

Yield: 10.3 mg of yellow solid (26%). R_f = 0.65 (ethyl acetate/hexanes 60:40). 1H -NMR (chloroform- d): δ = 3.00 (t, 4H), 3.78 (m, 4H), 4.82 (s, 2H), 7.10 (m, 4H), 7.49 (t, 2H), 7.58 (d, 1H), 7.78 (d, 1H). MS (ESI+): m/z = 410.19 $[M+H]^+$. ^{13}C -NMR (chloroform- d): δ = 166.092,

160.141, 158.292, 141.733, 141.407, 141.120, 133.741, 126.855, 125.940, 123.248, 121.695, 121.207, 112.841, 107.436, 68.741, 52.508, 51.856, 45.723, 42.408.

(4) 2-(3-chlorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 16.0 mg of yellow solid (44%). $R_f = 0.69$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.04$ (t, 4H), 3.74 (dt, 4H), 4.70 (s, 2H), 6.84 (d, 1H), 6.96 (dt, 2H), 7.12 (m, 2H), 7.19 (d, 1H), 7.50 (t, 1H), 7.78 (d, 1H). MS (ESI+): $m/z = 376.22$ $[\text{M}+\text{H}]^+$, 378.23 $[\text{M}+2+\text{H}]^+$.

(5) 2-(3-fluorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 15.6 mg of yellow solid (41%). $R_f = 0.69$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.04$ (t, 4H), 3.74 (dt, 4H), 4.70 (s, 2H), 6.7 (m, 3H), 7.12 (m, 2H), 7.35 (bs, 1H), 7.50 (t, 1H), 7.78 (d, 1H). MS (ESI+): $m/z = 360.21$ $[\text{M}+\text{H}]^+$.

(6) 1-(4-(2-nitrophenyl)piperazin-1-yl)-2-(3-(trifluoromethyl)phenoxy)ethanone

Yield: 11.1 mg of yellow solid (28%). $R_f = 0.76$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.04$ (t, 4H), 3.75 (dt, 4H), 4.76 (s, 2H), 7.13 (m, 4H), 7.34 (s, 1H), 7.40 (t, 1H), 7.50 (t, 1H), 7.79 (d, 1H). MS (ESI+): $m/z = 410.19$ $[\text{M}+\text{H}]^+$.

(7) 2-(4-chlorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 13.0 mg of yellow solid (36%). $R_f = 0.70$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.02$ (t, 4H), 3.74 (dt, 4H), 4.70 (s, 2H), 6.88 (dt, 2H), 7.12 (t, 2H), 7.24 (d, 2H), 7.50 (t, 1H), 7.79 (d, 1H). MS (ESI+): $m/z = 376.20$ $[\text{M}+\text{H}]^+$, 378.15 $[\text{M}+2+\text{H}]^+$.

(8) 2-(4-fluorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 12.2 mg of yellow solid (32%). $R_f = 0.60$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.02$ (t, 4H), 3.75 (dt, 4H), 4.68 (s, 2H), 6.89 (m, 2H), 6.97 (t, 2H), 7.12 (t, 2H), 7.50 (t, 1H), 7.79 (d, 1H). MS (ESI+): $m/z = 360.24$ $[\text{M}+\text{H}]^+$.

(9) 1-(4-(2-nitrophenyl)piperazin-1-yl)-2-(4-(trifluoromethyl)phenoxy)ethanone

Yield: 12.3 mg of yellow solid (31%). $R_f = 0.62$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.03$ (t, 4H), 3.75 (dt, 4H), 4.77 (s, 2H), 7.02 (d, 2H), 7.13 (t, 2H), 7.49 (d, 1H), 7.55 (d, 2H), 7.79 (d, 1H). MS (ESI+): $m/z = 410.20$ $[\text{M}+\text{H}]^+$. $^{13}\text{C-NMR}$ (chloroform- d): $\delta = 166.066, 160.227, 150.846, 146.716, 145.547, 133.750, 127.263, 127.225, 125.931, 123.439, 121.753, 114.748$ (2 carbons), 67.697, 52.738, 51.598, 45.618, 42.283.

(10) 2-(2,4-dichlorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 12.1 mg of yellow solid (30%). $R_f = 0.56$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.03$ (t, 4H), 3.76 (q, 4H), 4.78 (s, 2H), 6.95 (d, 1H), 7.10 (d, 2H), 7.18 (dd, 1H), 7.40 (d, 1H), 7.50 (t, 1H), 7.79 (d, 1H). MS (ESI+): $m/z = 410.24$ $[\text{M}+\text{H}]^+$, 412.19 $[\text{M}+2+\text{H}]^+$, 414.14 $[\text{M}+4+\text{H}]^+$.

(11) 2-(3,5-dichlorophenoxy)-1-(4-(2-nitrophenyl)piperazin-1-yl)ethanone

Yield: 7.2 mg of yellow solid (18%). $R_f = 0.56$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.06$ (t, 4H), 3.73 (dt, 4H), 4.69 (s, 2H), 6.85 (d, 2H), 7.00 (t, 1H), 7.14 (m,

2H), 7.52 (t, 1H), 7.80 (d, 1H). MS (ESI+): m/z = 410.09 [M+H]⁺, 412.15 [M+2+H]⁺, 414.10 [M+4+H]⁺.

(12) 2-(2-chlorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 10.1 mg of yellow solid (28%). R_f = 0.55 (ethyl acetate/hexanes 60:40). ¹H-NMR (chloroform-*d*): δ = 3.26 (dt, 4H), 3.83 (dt, 4H), 4.82 (s, 2H), 6.98 (m, 2H), 7.18 (t, 2H), 7.39 (t, 2H), 7.69 (m, 2H). MS (ESI+): m/z = 376.24 [M+H]⁺, 378.17 [M+2+H]⁺. ¹³C-NMR (chloroform-*d*): δ = 166.207, 152.791, 151.421, 139.788, 138.580, 129.994, 128.116, 122.692, 121.734, 114.690, 113.560, 110.426, 68.779, 49.154 (2 carbons), 45.235, 41.957.

(13) 2-(2-fluorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 14.2 mg of yellow solid (37%). R_f = 0.59 (ethyl acetate/hexanes 60:40). ¹H-NMR (chloroform-*d*): δ = 3.27 (m, 4H), 3.82 (q, 4H), 4.80 (s, 2H), 6.96 (m, 1H), 7.08 (m, 3H), 7.19 (d, 1H), 7.39 (t, 1H), 7.70 (m, 2H). MS (ESI+): m/z = 360.25 [M+H]⁺. ¹³C-NMR (chloroform-*d*): δ = 173.107, 166.255, 150.990, 149.332, 144.176, 130.119, 124.676, 122.663, 122.146, 116.731, 115.649, 115.284, 110.742, 69.201, 49.374, 48.953, 45.072, 41.775.

(14) 1-(4-(3-nitrophenyl)piperazin-1-yl)-2-(2-(trifluoromethyl)phenoxy)ethanone

Yield: 15.4 mg of yellow solid (39%). R_f = 0.75 (ethyl acetate/hexanes 60:40). ¹H-NMR (chloroform-*d*): δ = 3.23 (t, 4H), 3.81 (dt, 4H), 4.84 (s, 2H), 7.12 (m, 3H), 7.38 (t, 1H), 7.50 (t, 1H), 7.59 (d, 1H), 7.69 (m, 2H). MS (ESI+): m/z = 410.22 [M+H]⁺. ¹³C-NMR (chloroform-*d*): δ = 174.305, 170.625, 166.130, 133.808, 132.591, 130.061, 121.983, 121.341, 118.610, 115.064, 112.803, 110.666, 93.397, 68.789, 49.394, 48.723, 45.072, 41.852.

(15) 2-(3-chlorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 15.3 mg of yellow solid (42%). R_f = 0.58 (ethyl acetate/hexanes 60:40). ¹H-NMR (chloroform-*d*): δ = 3.27 (q, 4H), 3.78 (m, 4H), 4.72 (s, 2H), 6.85 (d, 1H), 6.96 (d, 2H), 7.18 (m, 2H), 7.40 (t, 1H), 7.70 (m, 2H). MS (ESI+): m/z = 376.20 [M+H]⁺, 378.14 [M+2+H]⁺. ¹³C-NMR (chloroform-*d*): δ = 166.427, 150.415, 150.309, 146.361, 130.646, 130.128, 122.270, 122.098, 115.342, 115.265, 112.898, 110.694, 10.005, 67.917, 49.298, 48.885, 41.718, 38.134.

(16) 2-(3-fluorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 10.0 mg of yellow solid (26%). R_f = 0.55 (ethyl acetate/hexanes 60:40). ¹H-NMR (chloroform-*d*): δ = 3.03 (t, 4H), 3.75 (dt, 4H), 4.70 (s, 2H), 6.69 (m, 3H), 7.12 (t, 2H), 7.35 (s, 1H), 7.50 (t, 1H), 7.79 (d, 1H). MS (ESI+): m/z = 360.17 [M+H]⁺. ¹³C-NMR (chloroform-*d*): δ = 172.235, 163.898, 162.537, 151.383, 130.722, 130.032, 122.222, 121.810, 114.815, 110.426, 110.254, 103.795, 102.827, 67.936, 49.001, 48.569, 45.062, 41.852.

(17) 1-(4-(3-nitrophenyl)piperazin-1-yl)-2-(3-(trifluoromethyl)phenoxy)ethanone

Yield: 10.8 mg of yellow solid (27%). R_f = 0.73 (ethyl acetate/hexanes 60:40). ¹H-NMR (chloroform-*d*): δ = 3.05 (t, 4H), 3.75 (dt, 4H), 4.76 (s, 2H), 7.13 (m, 4H), 7.34 (s, 1H), 7.41 (t, 1H), 7.50 (t, 1H), 7.79 (d, 1H). MS (ESI+): m/z = 410.28 [M+H]⁺.

(18) 2-(4-chlorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 10.7 mg of yellow solid (29%). R_f = 0.55 (ethyl acetate/hexanes 60:40). ¹H-NMR (chloroform-*d*): δ = 3.27 (q, 4H), 3.78 (q, 4H), 4.72 (s, 2H), 6.89 (d, 2H), 7.17 (d, 1H), 7.22 (s,

1H), 7.25 (s, 1H), 7.39 (t, 1H), 7.70 (m, 2H). MS (ESI+): $m/z = 376.22 [M+H]^+$, 378.22 $[M+2+H]^+$.

(19) 2-(4-fluorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 12.3 mg of yellow solid (32%). $R_f = 0.52$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform-*d*): $\delta = 3.26$ (q, 4H), 3.79 (q, 4H), 4.71 (s, 2H), 6.94 (m, 4H), 7.17 (d, 1H), 7.39 (t, 1H), 7.70 (m, 2H). MS (ESI+): $m/z = 360.24 [M+H]^+$. $^{13}\text{C-NMR}$ (chloroform-*d*): $\delta = 172.858$, 169.216, 158.953, 145.353, 145.240, 130.109, 116.386, 116.147, 115.812, 115.735, 110.656, 107.590, 104.945, 68.530, 63.873, 49.259, 46.615, 41.727.

(20) 1-(4-(3-nitrophenyl)piperazin-1-yl)-2-(4-(trifluoromethyl)phenoxy)ethanone

Yield: 12.4 mg of yellow solid (31%). $R_f = 0.66$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform-*d*): $\delta = 3.27$ (q, 4H), 3.79 (m, 4H), 4.79 (s, 2H), 7.03 (d, 2H), 7.17 (d, 1H), 7.40 (d, 1H), 7.40 (t, 1H), 7.56 (d, 2H), 7.70 (m, 2H). MS (ESI+): $m/z = 410.21 [M+H]^+$. $^{13}\text{C-NMR}$ (chloroform-*d*): $\delta = 173.068$, 166.782, 164.703, 156.452, 155.906, 152.197, 148.335, 145.633, 130.000, 130.166, 127.301, 124.311, 120.95, 114.719, 67.783, 51.828, 49.748, 49.374, 48.752.

(21) 2-(2,4-dichlorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 14.1 mg of yellow solid (35%). $R_f = 0.70$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform-*d*): $\delta = 3.26$ (m, 4H), 3.81 (m, 4H), 4.80 (s, 2H), 6.96 (d, 1H), 7.18 (m, 2H), 7.38 (m, 2H), 7.70 (m, 2H). MS (ESI+): $m/z = 410.14 [M+H]^+$, 412.11 $[M+2+H]^+$, 414.10 $[M+4+H]^+$.

(22) 2-(3,5-dichlorophenoxy)-1-(4-(3-nitrophenyl)piperazin-1-yl)ethanone

Yield: 10.3 mg of yellow solid (26%). $R_f = 0.61$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform-*d*): $\delta = 3.29$ (q, 4H), 3.77 (dt, 4H), 4.72 (s, 2H), 6.86 (d, 2H), 7.00 (t, 1H), 7.19 (d, 1H), 7.40 (t, 1H), 7.71 (m, 2H). MS (ESI+): $m/z = 410.09 [M+H]^+$, 412.19 $[M+2+H]^+$, 413.99 $[M+4+H]^+$. Purity (ELSD): >99%. $^{13}\text{C-NMR}$ (chloroform-*d*): $\delta = 165.546$, 158.781, 151.287, 149.342, 147.003, 135.763, 130.071, 122.385, 121.897, 144.978, 113.857 (2 carbons), 110.531, 67.764, 49.058, 48.646, 44.966, 41.804.

(23) 2-(2-chlorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 8.6 mg of yellow solid (24%). $R_f = 0.00$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform-*d*): $\delta = 3.24$ (m, 4H), 3.82 (dt, 4H), 4.81 (s, 2H), 6.81 (d, 2H), 6.98 (m, 2H), 7.20 (d, 1H), 7.37 (d, 1H), 8.12 (d, 2H). MS (ESI+): $m/z = 376.18 [M+H]^+$, 378.04 $[M+2+H]^+$.

(24) 2-(2-fluorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 7.1 mg of yellow solid (37%). $R_f = 0.17$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform-*d*): $\delta = 3.44$ (m, 4H), 3.83 (m, 4H), 4.80 (s, 2H), 6.86 (d, 2H), 6.96 (m, 1H), 7.08 (m, 3H), 8.14 (d, 2H). MS (ESI+): $m/z = 360.24 [M+H]^+$.

(25) 1-(4-(4-nitrophenyl)piperazin-1-yl)-2-(2-(trifluoromethyl)phenoxy)ethanone

Yield: 7.5 mg of yellow solid (19%). $R_f = 0.54$ (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform-*d*): $\delta = 3.39$ (t, 4H), 3.81 (dt, 4H), 4.84 (s, 2H), 6.80 (d, 2H), 7.09 (q, 2H), 7.50 (t, 1H), 7.59 (d, 1H), 8.12 (d, 2H). MS (ESI+): $m/z = 410.28 [M+H]^+$.

(26) 2-(3-chlorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 4.5 mg of yellow solid (12%). R_f = 0.00 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.43 (q, 4H), 3.78 (m, 4H), 4.70 (s, 2H), 6.83 (m, 3H), 6.94 (t, 1H), 6.98 (dq, 1H), 7.20 (d, 1H), 8.14 (d, 2H). MS (ESI+): m/z = 376.29 $[\text{M}+\text{H}]^+$, 378.23 $[\text{M}+2+\text{H}]^+$.

(27) 2-(3-fluorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 13.6 mg of yellow solid (35%). R_f = 0.19 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.43 (q, 4H), 3.78 (q, 4H), 4.72 (s, 2H), 6.70 (m, 3H), 6.81 (d, 2H), 7.23 (q, 1H), 8.12 (d, 2H). MS (ESI+): m/z = 360.23 $[\text{M}+\text{H}]^+$.

(28) 1-(4-(4-nitrophenyl)piperazin-1-yl)-2-(3-(trifluoromethyl)phenoxy)ethanone

Yield: 11.0 mg of yellow solid (28%). R_f = 0.41 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.45 (m, 4H), 3.80 (m, 4H), 4.78 (s, 2H), 6.84 (d, 2H), 7.15 (m, 2H), 7.28 (s, 1H), 7.42 (t, 1H), 8.14 (d, 2H). MS (ESI+): m/z = 410.28 $[\text{M}+\text{H}]^+$.

(29) 2-(4-chlorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 4.3 mg of yellow solid (12%). R_f = 0.49 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.42 (q, 4H), 3.80 (t, 4H), 4.72 (s, 2H), 6.87 (t, 4H), 7.24 (t, 2H), 8.14 (d, 2H). MS (ESI+): m/z = 376.30 $[\text{M}+\text{H}]^+$, 378.04 $[\text{M}+2+\text{H}]^+$.

(30) 2-(4-fluorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 4.5 mg of yellow solid (12%). R_f = 0.39 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.44 (q, 4H), 3.80 (t, 4H), 4.70 (s, 2H), 6.84 (d, 2H), 6.91 (d, 2H), 6.97 (d, 2H), 8.14 (d, 2H). MS (ESI+): m/z = 360.31 $[\text{M}+\text{H}]^+$.

(31) 1-(4-(4-nitrophenyl)piperazin-1-yl)-2-(4-(trifluoromethyl)phenoxy)ethanone

Yield: 7.1 mg of yellow solid (18%). R_f = 0.35 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.44 (q, 4H), 3.81 (q, 4H), 4.79 (s, 2H), 6.86 (d, 2H), 7.02 (d, 2H), 7.56 (d, 2H), 8.15 (d, 2H). MS (ESI+): m/z = 410.28 $[\text{M}+\text{H}]^+$.

(32) 2-(2,4-dichlorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 5.8 mg of yellow solid (15%). R_f = 0.00 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.42 (m, 4H), 3.81 (dt, 4H), 4.80 (s, 2H), 6.81 (d, 2H), 6.95 (d, 1H), 7.19 (dd, 1H), 7.38 (d, 1H), 8.13 (d, 2H). MS (ESI+): m/z = 410.25 $[\text{M}+\text{H}]^+$, 412.20 $[\text{M}+2+\text{H}]^+$, 414.25 $[\text{M}+4+\text{H}]^+$.

(33) 2-(3,5-dichlorophenoxy)-1-(4-(4-nitrophenyl)piperazin-1-yl)ethanone

Yield: 5.2 mg of yellow solid (13%). R_f = 0.00 (ethyl acetate/hexanes 60:40). $^1\text{H-NMR}$ (chloroform- d): δ = 3.46 (q, 4H), 3.77 (dt, 4H), 4.71 (s, 2H), 6.81 (s, 1H), 6.84 (t, 1H), 6.85 (d, 2H), 7.00 (t, 1H), 8.14 (d, 2H). MS (ESI+): m/z = 410.28 $[\text{M}+\text{H}]^+$, 412.25 $[\text{M}+2+\text{H}]^+$, 414.25 $[\text{M}+4+\text{H}]^+$.

2.8.2. General Procedure for Carboxy Analog Synthesis: Method A for Ortho and Meta Analogs

Twenty-two carboxy-containing analogs of GRI392104 (table 1) were synthesized using a two-step reaction (scheme 2). To a dry round bottom flask, 140 μmol of 2-phenoxyacetic acid building block, 126 μmol of HBTU, and 140 μmol DIPEA were combined in 4 mL of

dichloromethane (DCM) and sonicated for 5 minutes before transferring 140 μ mol of ortho- or meta-carboxy amine building block into the reaction flask with an additional 1 mL of DCM. This was sonicated for 60 minutes and tracked with analytical TLC in either 95:5 ethyl acetate/methanol (ortho compounds) or 90:10 chloroform/methanol (meta compounds). IPProducts were then extracted into DCM and washed with additional water three times before drying the organic layer over sodium sulfate and filtering into a tared flask and drying to a white crude powder to confirm synthesis via ^1H -NMR (in CDCl_3). Once synthesis was confirmed, crude product was mixed with silica and additional chloroform before drying the resulting slurry *in vacuo* and utilizing flash chromatography to purify the compounds over a 10g Biotage SNAP column at 25 mL/min unless otherwise mentioned (eluent were the same from analytical TLC). Purity was confirmed by ^1H -NMR before drying the products to a solid white residue (supplemental figure 1). ^{13}C -NMR data was collected for all previously unreported compounds (supplemental figure 2). High resolution MS data for all previously unreported compounds gave the expected mass values with absolute errors ranging from 0.0 to 20.3 ppm. They were then diluted to 3 mM in DMSO for activity analysis.

2.8.3. General Procedure for Carboxy Analog Synthesis: Method B for Para Analogs

For the remaining eleven para-substituted carboxy-containing analogs of GRI392104 (table 1), the same reagents were combined in a clean, dry flask in 1 mL dimethyl sulfoxide (DMSO). After 5 minutes of stirring, the clear solution turned amber and 140 μ mol of solid para-carboxy amine building block was dissolved into the reaction mixture. Over the course of an hour, the amber solution slowly turned cloudy white. The reactions were worked up in the same manner as the other twenty-two carboxy analogs, except ethyl acetate was used instead of methylene chloride before synthesis was confirmed with ^1H -NMR. Products were then purified over 10 g Biotage SNAP columns at 25 mL/min (method C) or via preparative TLC in 92.5:7.5 chloroform/methanol (method D). Products from method D were extracted in 50:50 ethyl acetate/methylene chloride and dried to a solid white residue. Purity was confirmed via ^1H -NMR before drying the products to a solid white powder (supplemental figure 1). ^{13}C -NMR data was collected for all previously unreported compounds (supplemental figure 2). High resolution MS data for all previously unreported compounds gave the expected mass values with absolute errors ranging from 0.0 to 20.3 ppm. Compounds were stored at 3 mM in DMSO for activity analysis.

(34) 2-(4-(2-(2-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 21.8 mg of white solid (46%). Retention time = 1.8 min (method A, 95:5 ethyl acetate/methanol). ^1H -NMR (dimethyl sulfoxide-*d*): δ = 3.09 (dt, 4H), 3.68 (m, 4H), 5.04 (s, 2H), 6.96 (t, 1H), 7.08 (d, 1H), 7.31 (m, 2H), 7.43 (dd, 1H), 7.53 (d, 1H), 7.64 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 373.13 [$\text{M}-\text{H}$] $^-$, 375.13 [$\text{M}+2-\text{H}$] $^-$. ^{13}C -NMR (dimethyl sulfoxide-*d*): δ = 167.549, 166.169, 153.903, 151.028, 134.210, 131.518, 130.464, 128.604, 126.429, 125.356, 122.854, 122.270, 121.609, 114.489, 66.719, 52.920, 52.527, 44.813, 41.948.

(35) 2-(4-(2-(2-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 13.1 mg of white solid (29%). Retention time = 1.7 min (method A, 95:5 ethyl acetate/methanol). ^1H -NMR (dimethyl sulfoxide-*d*): δ = 3.08 (dt, 4H), 3.67 (t, 4H), 5.00 (s, 2H), 6.95 (m, 1H), 7.10 (s, 1H), 7.12 (t, 1H), 7.22 (q, 1H), 7.34 (t, 1H), 7.54 (d, 1H), 7.63 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 357.15 [$\text{M}-\text{H}$] $^-$. ^{13}C -NMR (dimethyl sulfoxide-*d*): δ = 167.530,

166.265, 157.180, 151.076, 134.134, 131.508, 126.324, 125.442, 125.078, 125.059, 122.836, 121.743, 116.425, 115.687, 66.662, 52.882, 52.456, 44.727, 41.929.

(36) 2-(4-(2-(2-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 13.7 mg of white solid (27%). Retention time = 1.8 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.08 (dt, 4H), 3.67 (t, 4H), 5.10 (s, 2H), 7.10 (t, 1H), 7.17 (d, 1H), 7.34 (t, 1H), 7.52 (d, 1H), 7.62 (m, 3H), 7.95 (d, 1H). MS (ESI-): m/z = 407.14 [M-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ =

(37) 2-(4-(2-(3-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 19.0 mg of white solid (40%). Retention time = 1.8 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.08 (dt, 4H), 3.67 (t, 4H), 4.95 (s, 2H), 6.94 (d, 1H), 7.01 (d, 1H), 7.08 (t, 1H), 7.34 (q, 2H), 7.54 (d, 1H), 7.63 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 373.13 [M-H]⁻, 375.13 [M+2-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 167.520, 166.303, 159.557, 151.076, 134.162, 134.124, 131.518, 131.268, 126.362, 125.404, 122.874, 121.341, 115.179, 114.441, 66.307, 52.853, 52.565, 44.708, 41.909.

(38) 2-(4-(2-(3-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 12.5 mg of white solid (28%). Retention time = 1.7 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.09 (dt, 4H), 3.67 (t, 4H), 4.93 (s, 2H), 6.83 (m, 3H), 7.33 (m, 2H), 7.55 (d, 1H), 7.64 (d, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 357.14 [M-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 167.510, 166.303, 160.141, 151.086, 134.153, 131.508, 131.125, 126.353, 125.404, 122.874, 111.681, 108.078, 102.865, 102.616, 66.403, 52.863, 52.575, 44.717, 41.909.

(39) 2-(4-(2-(3-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 14.7 mg of white solid (29%). Retention time = 1.6 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.10 (dt, 4H), 3.68 (t, 4H), 5.03 (s, 2H), 7.27 (d, 2H), 7.34 (t, 2H), 7.54 (m, 2H), 7.63 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 407.16 [M-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 167.577, 166.313, 158.876, 150.980, 134.239, 131.527, 131.134, 126.506, 125.308, 124.465, 122.855, 119.443, 117.977, 111.863, 111.336, 66.230, 52.882, 52.585, 44.698, 41.900.

(40) 2-(4-(2-(4-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 10.7 mg of white solid (23%). Retention time = 1.7 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.08 (dt, 4H), 3.67 (t, 4H), 4.92 (s, 2H), 6.99 (d, 2H), 7.34 (m, 3H), 7.54 (d, 1H), 7.63 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 373.13 [M-H]⁻, 375.14 [M+2-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 167.530, 166.428, 157.449, 151.038, 134.191, 131.508, 129.620, 126.420, 125.356, 125.059, 122.884, 116.971, 66.374, 52.872, 52.565, 44.727, 41.900.

(41) 2-(4-(2-(4-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 8.2 mg of white solid (18%). Retention time = 1.8 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.08 (dt, 4H), 3.67 (t, 4H), 4.88 (s, 2H), 6.97 (m, 2H), 7.13 (t, 2H), 7.34 (t, 1H), 7.54 (d, 1H), 7.63 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 357.16 [M-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 172.072, 167.510, 166.600, 151.622,

151.076, 140.200, 134.134, 131.508, 125.423, 122.864, 116.521, 116.434, 116.339, 116.118, 62.263, 57.395, 52.575, 44.736, 43.385.

(42) 2-(4-(2-(4-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 16.2 mg of white solid (31%). Retention time = 1.6 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.10 (dt, 4H), 3.67 (t, 4H), 5.05 (s, 2H), 7.15 (d, 2H), 7.35 (t, 1H), 7.55 (d, 1H), 7.64 (m, 3H), 7.95 (d, 1H). MS (ESI-): m/z = 407.15 [M-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 167.520, 166.150, 163.629, 161.521, 160.247, 158.014, 151.095, 134.143, 131.518, 127.340, 126.314, 125.413, 122.845, 115.725 (2 carbons), 66.173, 52.863, 52.546, 44.708, 41.938.

(43) 2-(4-(2-(2,4-dichlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 11.1 mg of white solid (22%). Retention time = 1.7 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.09 (dt, 4H), 3.66 (t, 4H), 5.08 (s, 2H), 7.11 (d, 1H), 7.35 (m, 2H), 7.54 (d, 1H), 7.59 (d, 1H), 7.64 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 407.11 [M-H]⁻, 409.11 [M+2-H]⁻, 4011.15 [M+4-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 167.558, 165.910, 153.165, 151.018, 146.371, 134.210, 131.527, 129.754, 128.346, 126.429, 125.346, 122.836, 122.654, 115.764, 66.853, 53.457, 52.853, 44.698, 41.948.

(44) 2-(4-(2-(3,5-dichlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 19.8 mg of white solid (38%). Retention time = 1.5 min (method A, 95:5 ethyl acetate/methanol). ¹H-NMR (dimethyl sulfoxide-*d*): δ = 3.09 (dt, 4H), 3.66 (m, 4H), 5.01 (s, 2H), 7.10 (d, 2H), 7.17 (t, 1H), 7.34 (t, 1H), 7.54 (d, 1H), 7.64 (t, 1H), 7.95 (d, 1H). MS (ESI-): m/z = 407.09 [M-H]⁻, 409.10 [M+2-H]⁻, 411.11 [M+4-H]⁻. ¹³C-NMR (dimethyl sulfoxide -*d*): δ = 169.082, 167.510, 165.977, 160.160, 151.086, 134.958, 134.162, 131.518, 126.333, 125.404, 122.836, 121.092, 114.690 (2 carbons), 66.508, 52.834, 52.537, 44.641, 41.919.

(45) 3-(4-(2-(2-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 12.3 mg of white solid (26%). Retention time = 3.5 min (method A with 20 mL/min flow rate, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.22 (dt, 4H), 3.81 (dt, 4H), 4.82 (s, 2H), 6.93 (t, 1H), 7.02 (d, 1H), 7.12 (d, 1H), 7.20 (d, 1H), 7.36 (d, 2H), 7.61 (s, 2H). MS (ESI-): m/z = 373.15 [M-H]⁻, 375.16 [M+2-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 166.198, 164.147, 156.414, 155.062, 154.210, 153.759, 136.568, 136.491, 130.684, 129.687, 128.125, 122.692, 120.018, 133.560, 68.770, 49.106, 47.784, 45.033, 41.871.

(46) 3-(4-(2-(2-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 20.7 mg of white solid (46%). Retention time = 2.5 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.21 (m, 4H), 3.79 (m, 4H), 4.81 (s, 2H), 6.94 (m, 1H), 7.06 (m, 3H), 7.12 (d, 1H), 7.34 (t, 1H), 7.62 (s, 2H). MS (ESI-): m/z = 357.18 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 166.313, 165.373, 156.107, 130.396, 129.572, 124.676, 124.637, 122.654, 122.577, 122.021, 118.006, 116.703, 116.530, 115.706, 69.134, 49.384, 45.263, 41.977, 40.788.

(47) 3-(4-(2-(2-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 5.0 mg of white solid (10%). Retention time = 2.0 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.20 (t, 4H), 3.82 (dt, 4H), 4.85 (s, 2H),

7.05 (t, 1H), 7.14 (t, 2H), 7.36 (t, 1H), 7.50 (t, 1H), 7.61 (m, 3H). MS (ESI-): m/z = 407.17 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 168.881, 166.178, 164.204, 162.968, 155.819, 155.369, 153.548, 150.674, 133.779, 129.620, 125.385, 124.369, 121.293, 118.169, 112.831, 68.751, 50.189, 49.451, 45.177, 38.728.

(48) 3-(4-(2-(3-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 10.1 mg of white solid (21%). Retention time = 1.3 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.24 (q, 4H), 3.79 (dt, 4H), 4.73 (s, 2H), 6.86 (d, 1H), 6.97 (d, 2H), 7.20 (d, 2H), 7.37 (t, 1H), 7.64 (m, 2H). MS (ESI-): m/z = 373.15 [M-H]⁻, 375.16 [M+2-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 167.252, 166.226, 158.464, 145.518, 134.057, 130.617, 130.454, 129.639, 122.213, 118.102, 118.006, 115.361, 112.927, 106.727, 69.594, 45.091, 45.062, 41.852, 40.788.

(49) 3-(4-(2-(3-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 8.1 mg of white solid (18%). Retention time = 2.0 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.24 (q, 4H), 3.79 (dt, 4H), 4.73 (s, 2H), 6.72 (dd, 3H), 7.19 (dd, 2H), 7.37 (t, 1H), 7.64 (m, 2H). MS (ESI-): m/z = 357.19 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 169.638, 166.313, 162.441, 130.722, 130.626, 130.473, 129.697, 125.969, 118.955, 118.198, 110.254, 108.797, 102.865, 102.616, 67.936, 50.199, 45.024, 43.816, 41.785.

(50) 3-(4-(2-(3-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 5.0 mg of white solid (10%). Retention time = 1.5 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.25 (q, 4H), 3.80 (dt, 4H), 4.78 (s, 2H), 7.16 (m, 3H), 7.27 (s, 1H), 7.40 (q, 2H), 7.65 (m, 2H). MS (ESI-): m/z = 407.18 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 172.235, 166.169, 166.015, 157.669, 132.457, 130.435, 130.339, 129.687, 118.859, 118.820, 118.121, 118.025, 117.891, 111.875, 111.835, 64.927, 45.110, 43.893, 42.513, 41.929.

(51) 3-(4-(2-(4-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 5.5 mg of white solid (12%). Retention time = 1.8 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.23 (q, 4H), 3.79 (m, 4H), 4.72 (s, 2H), 6.89 (d, 2H), 7.17 (d, 1H), 7.24 (d, 2H), 7.37 (t, 1H), 7.64 (m, 2H). MS (ESI-): m/z = 373.16 [M-H]⁻, 375.17 [M+2-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 171.401, 168.660, 166.380, 158.857, 154.440, 139.586, 134.431, 131.278, 129.774, 129.687, 125.902, 119.664, 116.089, 115.237, 67.697, 46.279, 43.644, 42.015, 38.048.

(52) 3-(4-(2-(4-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 7.4 mg of white solid (16%). Retention time = 1.8 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.19 (m, 4H), 3.75 (m, 4H), 4.70 (s, 2H), 6.91 (d, 2H), 6.97 (t, 2H), 7.11 (d, 1H), 7.33 (t, 1H), 7.60 (m, 2H). MS (ESI-): m/z = 357.21 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 170.059, 166.667, 162.374, 162.058, 156.049, 153.855, 130.454, 130.071, 129.678, 118.159, 116.367, 116.137, 115.831, 115.754, 68.434, 50.112, 45.005, 41.794, 40.807.

(53) 3-(4-(2-(4-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 9.9 mg of white solid (19%). Retention time = 2.5 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.25 (m, 4H), 3.80 (dt, 4H), 4.79 (s, 2H), 7.03 (d, 2H), 7.18 (d, 1H), 7.37 (t, 1H), 7.56 (d, 2H), 7.65 (m, 2H). MS (ESI-): m/z = 407.19 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 170.433, 166.025, 163.802, 161.866, 160.208, 133.894, 129.563, 127.253, 121.916, 119.884, 117.891, 116.741, 114.738, 109.804, 67.677, 45.206, 43.701, 42.005, 40.836.

(54) 3-(4-(2-(2,4-dichlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 7.8 mg of white solid (15%). Retention time = 2.5 min (method A with flow rate 20 mL/min, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.24 (dt, 4H), 3.81 (m, 4H), 4.81 (s, 2H), 6.97 (d, 1H), 7.17 (t, 2H), 7.38 (q, 2H), 7.63 (m, 2H). MS (ESI-): m/z = 407.13 [M-H]⁻, 409.14 [M+2-H]⁻, 411.15 [M+4-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 170.404, 165.814, 161.780, 160.544, 146.927, 146.256, 144.339, 138.254, 130.396, 129.620, 127.972, 126.812, 114.412, 111.969, 68.885, 45.283, 42.111, 41.986, 38.546.

(55) 3-(4-(2-(3,5-dichlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 6.1 mg of white solid (12%). Retention time = 2.0 min (method A, 90:10 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.26 (q, 4H), 3.78 (dt, 4H), 4.72 (s, 2H), 6.87 (d, 2H), 7.00 (t, 1H), 7.19 (d, 1H), 7.38 (t, 1H), 7.65 (m, 2H). MS (ESI-): m/z = 407.12 [M-H]⁻, 409.14 [M+2-H]⁻, 411.16 [M+4-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 170.634, 165.623, 158.800, 155.369, 135.753, 130.454, 129.668, 123.804, 123.008, 122.376, 122.261, 118.140, 117.651, 113.876, 67.716, 50.007, 49.566, 45.033, 41.881.

(56) 4-(4-(2-(2-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 17.9 mg of white solid (38%). Retention time = 1.8 min (method B and C, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.36 (m, 4H), 3.18 (dt, 4H), 4.82 (s, 2H), 6.86 (d, 2H), 6.94 (t, 1H), 7.02 (d, 1H), 7.21 (d, 1H), 7.37 (d, 1H), 7.98 (d, 2H). MS (ESI-): m/z = 373.17 [M-H]⁻, 375.19 [M+2-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 172.340, 166.955, 163.265, 160.496, 159.231, 155.235, 153.567, 151.162, 147.885, 132.121, 130.693, 115.524, 114.278, 113.512, 64.841, 56.392, 51.262, 46.203, 40.980.

(57) 4-(4-(2-(2-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 7.4 mg of white solid (16%). R_f = 0.46 (method B and D, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.37 (m, 4H), 3.80 (q, 4H), 4.80 (s, 2H), 6.88 (d, 2H), 6.96 (m, 1H), 7.08 (m, 3H), 7.98 (d, 2H). MS (ESI-): m/z = 357.17 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 167.041, 165.106, 160.908, 155.369, 138.590, 135.389, 132.160, 127.598, 127.090, 126.477, 122.644, 116.540, 114.575, 113.339, 69.182, 53.984, 46.337, 45.829, 44.573.

(58) 4-(4-(2-(2-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 4.2 mg of white solid (8%). R_f = 0.32 (method B and D, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.28 (dt, 4H), 3.80 (dt, 4H), 4.84 (s, 2H), 6.85 (d, 2H), 7.06 (t, 1H), 7.13 (d, 1H), 7.50 (t, 1H), 7.59 (d, 1H), 7.98 (d, 2H). MS (ESI-): m/z = 407.19 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 170.788, 167.204, 166.198, 153.826, 148.124, 133.808, 132.140, 130.655, 129.467, 127.483, 121.331, 119.923, 117.987, 114.345, 112.793, 68.770, 48.282, 47.630, 44.957, 41.852.

(59) 4-(4-(2-(3-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 13.9 mg of white solid (29%). Retention time = 3.3 min (method B and C, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.32 (dt, 4H), 3.78 (dt, 4H), 4.72 (s, 2H), 6.86 (m, 3H), 6.95 (m, 1H), 6.99 (s, 1H), 7.20 (d, 1H), 7.99 (d, 2H). MS (ESI-): m/z = 373.22 [M-H]⁻, 375.13 [M+2-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 166.236, 166.054, 160.371, 147.080, 142.384, 132.140, 130.626, 123.545, 122.242, 119.779, 117.699, 115.352, 114.240, 112.879, 67.860, 50.946, 50.141, 48.033, 47.535.

(60) 4-(4-(2-(3-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 3.7 mg of white solid (8%). R_f = 0.46 (method B and D, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.36 (q, 4H), 3.77 (m, 4H), 4.73 (s, 2H), 6.66 (m, 2H), 6.74 (d, 1H), 6.86 (d, 2H), 7.21 (d, 1H), 7.98 (d, 2H). MS (ESI-): m/z = 357.20 [M-H]⁻. ¹³C-NMR (dimethyl sulfoxide-*d*): δ = 167.760, 166.188, 155.829, 155.034, 150.999, 140.602, 131.412, 131.086, 123.717, 120.267, 114.096, 111.624, 108.049, 107.839, 66.489, 47.295, 47.055, 44.046, 42.925.

(61) 4-(4-(2-(3-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 2.0 mg of white solid (4%). R_f = 0.45 (method B and D, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.70 (m, 4H), 3.76 (m, 4H), 4.77 (s, 2H), 6.86 (d, 2H), 7.15 (m, 2H), 7.27 (s, 1H), 7.41 (t, 1H), 7.97 (d, 2H). MS (ESI-): m/z = 407.22 [M-H]⁻. ¹³C-NMR (dimethyl sulfoxide-*d*): δ = 167.760, 166.054, 165.115, 157.937, 154.947, 153.884, 152.839, 149.629, 144.905, 131.422, 131.086, 120.267, 119.712, 119.443, 114.106, 66.336, 49.509, 47.218, 46.845, 43.645.

(62) 4-(4-(2-(4-chlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 7.7 mg of white solid (16%). Retention time = 2.0 min (method B and C, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.36 (q, 4H), 3.77 (m, 4H), 4.72 (s, 2H), 6.88 (t, 4H), 7.24 (d, 2H), 7.98 (d, 2H). MS (ESI-): m/z = 373.12 [M-H]⁻, 375.21 [M+2-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 169.571, 166.485, 163.869, 160.764, 156.346, 153.951, 145.346, 134.747, 133.942, 132.160, 129.726, 126.966, 115.955, 114.221, 68.070, 53.897, 48.023, 44.918, 41.747.

(63) 4-(4-(2-(4-fluorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 12.4 mg of white solid (27%). R_f = 0.50 (method B and D, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.36 (m, 4H), 3.78 (m, 4H), 4.70 (s, 2H), 6.88 (d, 2H), 6.91 (d, 2H), 6.96 (d, 2H), 7.99 (d, 2H). MS (ESI-): m/z = 357.18 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 170.951, 166.686, 153.922, 153.855, 132.150, 130.521, 125.212, 120.826, 116.367, 116.137, 115.802, 115.725, 114.240, 113.847, 68.763, 48.062, 47.573, 44.909, 41.718.

(64) 4-(4-(2-(4-(trifluoromethyl)phenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 6.6 mg of white solid (13%). R_f = 0.32 (method B and D, 92.5:7.5 chloroform/methanol). ¹H-NMR (chloroform-*d*): δ = 3.37 (d, 4H), 3.78 (m, 4H), 4.79 (s, 2H), 6.87 (d, 2H), 7.03 (d, 2H), 7.56 (d, 2H), 7.99 (d, 2H). MS (ESI-): m/z = 407.19 [M-H]⁻. ¹³C-NMR (chloroform-*d*): δ = 172.311, 166.667, 166.083, 159.672, 156.759, 154.775, 136.050, 132.140, 131.067, 127.272, 126.592, 122.040, 120.833, 114.709, 114.345, 67.725, 44.899, 41.718, 39.447, 38.421.

(65) 4-(4-(2-(2,4-dichlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 4.8 mg of white solid (9%). $R_f = 0.45$ (method B and D, 92.5:7.5 chloroform/methanol). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.36$ (m, 4H), 3.80 (m, 4H), 4.80 (d, 2H), 6.87 (d, 2H), 6.96 (d, 1H), 7.18 (dd, 1H), 7.38 (d, 1H), 7.99 (d, 2H). MS (ESI-): $m/z = 407.11$ $[\text{M-H}]^-$, 409.14 $[\text{M}+2\text{-H}]^-$, 411.21 $[\text{M}+4\text{-H}]^-$. $^{13}\text{C-NMR}$ (dimethyl sulfoxide- d): $\delta = 171.938$, 167.760, 151.823, 149.677, 146.448, 145.911, 135.763, 131.422, 129.745, 128.336, 125.078, 122.634, 115.764, 114.096, 66.959, 49.144, 47.247, 44.267, 44.027.

(66) 4-(4-(2-(3,5-dichlorophenoxy)acetyl)piperazin-1-yl)benzoic acid

Yield: 5.9 mg of white solid (11%). $R_f = 0.36$ (method B and D, 92.5:7.5 chloroform/methanol). $^1\text{H-NMR}$ (chloroform- d): $\delta = 3.39$ (m, 4H), 3.77 (dt, 4H), 4.71 (s, 2H), 6.86 (s, 2H), 6.89 (d, 2H), 7.00 (t, 1H), 7.99 (d, 2H). MS (ESI-): $m/z = 407.15$ $[\text{M-H}]^-$, 409.22 $[\text{M}+2\text{-H}]^-$, 411.34 $[\text{M}+4\text{-H}]^-$. $^{13}\text{C-NMR}$ (chloroform- d): $\delta = 171.219$, 165.929, 158.809, 148.566, 146.016, 145.393, 141.254, 135.724, 132.246, 122.500, 114.020, 113.914, 113.837, 113.521, 64.946, 46.777, 46.730, 45.580, 44.976.

3. Results

3.1. Protein Model Development

Sequences of both mouse (PDB[33] ID: 3NKM[27]) and rat (PDB ID: 2XR9[26]) ATX were aligned with the FASTA sequence of human ATX (GenBank[34] ID: Q13822) using MOE (Molecular Operating Environment, Chemical Computing Group, Montreal, Canada). This analysis confirmed that both mouse and rat ATX have 95% sequence identity with human ATX. Due to the high similarities either crystal structure is an appropriate surrogate for the human homologue for modeling. It is worth noting that the currently available human ATX structures (PDB ID: 4ZG6, 4ZG7, 4ZG9, and 4ZGA) were published after this modeling was developed.[30] However, the mouse and rat crystal structures were not without limitations. For example, the rat ATX structure was missing portions of the backbone distant from the active site and hydrophobic regions, whereas the mouse structure contained several sidechains truncated to alanine (methyl) due to unresolved atomic positions (K59, E67, K104, R162, R244, R246, F274, N398, L458, K462, R549, Q559, R602, E642, and K666). All truncated side chain atoms were reconstructed to their proper form using the structure preparation feature within MOE (Molecular Operating Environment, Chemical Computing Group, Montreal, Canada) and, after alignment and minimization, were similar in orientation to structures that included these atoms (such as PDB ID: 3WAV[28], all atom RMSD 0.48 Å and alpha carbon RMSD of 0.19 Å).

3.2. Pharmacophore Design

Previously, two potent inhibitors (KM04131 and PF8380) had been docked into the mouse ATX crystal structure (PDB ID: 3NKM), (figure 2)[22]. The modeled positions of these two hydrophobic tunnel targeted ATX inhibitors were used to generate a structure-based pharmacophore for ATX inhibition (figure 2). An exclusion volume shape, with a radius of 4 Å, was added to the pharmacophore in order to define the surface of the receptor. Pharmacophore metrics were calculated for this model using an internal database of 457[22] compounds of

known ATX activity (equations 1-5).[37] This pharmacophore selected 5 out of 111 actives and 5 out of 346 inactives in that database, resulting in a 50% yield of actives (number of actives found versus total number of compounds, equation 1). Other metrics analyzed were sensitivity, specificity, accuracy, and enrichment (equations 2-5). Abbreviations found in the equations below include: true positives (TP), false positives (FP), false negatives (FN), true negatives (TN), number of compounds found by the model (n), total entries in the database (N), and the number of actives (A). The true positives and false positives are compounds found by the pharmacophore which are either active or inactive, respectively, whereas true negatives and false negatives are compounds which are not found by the pharmacophore but are inactive or active compounds for ATX inhibition, respectively. The total number of actives (A) is 111 whereas the total number (N) of entries in the test database is 457 compounds. For yield of actives (equation 1), sensitivity (equation 2), specificity (equation 3), and accuracy (equation 4) results should be close to 1. However, enrichment (equation 5) should be above 1 for optimal results. Although this pharmacophore showed low sensitivity (equation 2), it had high specificity and was used to search the Genomic Research Institute (GRI) database from the University of Cincinnati Drug Discovery Center (UC-DDC). A total of 2,090 out of 342,420 compounds were identified as potential hits. For the purposes of this study, active compounds inhibit ATX activity by 50% or better at 10 μ M.

$$\text{yield of actives} = \frac{\text{TP}}{n} = 0.50 \quad (1)$$

$$\text{sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FN}} = 0.045 \quad (2)$$

$$\text{specificity} = \frac{\text{TN}}{\text{TN} + \text{FP}} = 0.99 \quad (3)$$

$$\text{accuracy} = \frac{\text{TP} + \text{TN}}{N} = 0.76 \quad (4)$$

$$\text{enrichment} = \frac{\text{TP}/n}{A/N} = 2.06 \quad (5)$$

Figure 2: Creation of a pharmacophore from KM04131 and PF8380. A) Two-dimensional structures of known inhibitors, KM04131 and PF8380[36]. B) KM04131 (black) and PF8380 (teal) docked in mouse autotaxin. C) Overlay of pharmacophore (orange and green spheres with blue outline) with the docked compounds. The orange spheres represent groups which can be aromatic or hydrophobic whereas the green sphere is only hydrophobic. D) Pharmacophore alone with exclusion volume shape shown in blue outline.

3.3. Candidate Inhibitor Modeling

The 2,090 GRI compounds selected as matches to the pharmacophore search of the GRI database were docked into the 3NKM crystal structure using AutoDock Vina.[38] Once docked, the exclusion volume shape narrowed the results to 288 compounds that were targeted to the same volume as the starting actives, KM04131 and PF8380 (figures 2 and 3). Lipinski's rules[32] were used to filter the results further to 192 drug-like compounds. In order to sample a wide variety of compounds, the Tanimoto coefficient was calculated on the basis of MACCS structural keys to organize these 192 compounds into 72 clusters (figure 4) with $\geq 55\%$ similarity (or greater). One representative compound from each cluster was ordered for *in vitro* screening.

Figure 3: Docked ligands before (left) and after (right) utilizing the exclusion volume shape. All 2090 compounds selected by the pharmacophore from the GRI database were docked into autotaxin (left). The hit-list was narrowed to compounds that fit into the region of interest with the exclusion volume shape, resulting in 288 compounds (right).

Figure 4: Example clusters obtained from utilizing the Tanimoto coefficient on the basis of MACCS structural keys. Some compounds clustered into groups of two or more (like the representatives in Cluster A showing 2 of 85 compounds and Cluster B showing 2 of 4 compounds) while others were unique in the database and had no other compounds which were within 55% similarity (such as Cluster C).

3.4. *In Vitro* Screening

None of the 72 compounds showed assay interference in the form of independent fluorescence or absorbance or suppression of signals from carboxyfluorescein (primary activity assay) or *para*-nitrophenolate (secondary activity assay) (data not shown, structures supplemental figure 3). Because of this, all 72 compounds were assayed first against FS-3, then against pNP-TMP. Compounds exhibiting 50% inhibition or greater at 10 μ M with either substrate were considered

an active inhibitor of ATX (table 2, supplemental figure 4). Initially there appeared to be five active compounds, but upon retesting, the initial response to GRI 792685 was determined to be a false positive (supplemental figure 4). The IC_{50} values for the 4 active compounds were determined in a dose response assay with FS-3 (table 2). Since none of the four compounds showed 50% inhibition or greater of ATX-catalyzed pNP-TMP hydrolysis at 10 μ M dose responses were not determined for the secondary substrate. This differential inhibition pattern suggested that these inhibitors likely prefer the hydrophobic region of ATX (which is accessed by the larger substrate FS-3) and are distant from the active site (where the smaller substrate pNP-TMP is localized).[43] Because GRI 392104 (IC_{50} 4 μ M) was the most potent inhibitor, was tractable to straightforward synthetic approaches, and also from a single compound cluster, analogs were designed to improve ATX inhibitory activity and explore the structure-activity relationship.

Table 2: Top GRI compounds

GRI Compound	FS-3 Inhibition (%)	FS-3 IC ₅₀ (μM)	pNP-TMP Inhibition (%)
392104	65.5 ± 3.2	4.0 ± 0.3	27.7 ± 2.0
519441	59.7 ± 1.7	5.7 ± 0.8	-45.4 ± 2.5
96880	54.1 ± 0.2	6.0 ± 0.5	-6.9 ± 2.0
93694	48.0 ± 4.4	6.6 ± 0.9	7.5 ± 2.4

3.5. GRI 392104 Analog Synthesis and Activity Screening

A straightforward two-step approach (scheme 2) yielded sixty-six analogs of GRI392104 (table 2). Synthesis of these compounds was confirmed by ¹H-NMR before preparative TLC or flash chromatography was used for purification. Liquid chromatography Mass spectrometry, and ¹H-NMR and ¹³C-NMR were used to confirm the identity and purity of all synthetic targets.

The 66 compounds in table 2 were tested against purified ATX in presence of either FS-3 (primary active assay) or pNP-TMP (secondary activity assay) to determine ATX inhibition (tables 3 and 4). None of these analogs showed auto-fluorescence or absorbance at tested wavelengths (data not shown). Of these 66 compounds, 36 were inhibitors of ATX, showing 50% inhibition or greater against FS-3 at 10 μM. Compound **22** showed significantly improved potency over GRI392104, with an IC₅₀ of 670 nM, a six-fold improvement over the 4 μM potency of GRI392104 (tables 3 and 4). Compounds which inhibit pNP-TMP hydrolysis may

bind closer to the active site instead of in the targeted hydrophobic pocket or they may elicit allosteric effects from the hydrophobic pocket. Some compounds inhibit both substrates with similar potencies (such as **55**), suggesting they might occupy a binding site closer to the active site. However, other compounds have drastically decreased potencies against pNP-TMP in comparison to potency against FS-3 as the substrate (such as **22**), indicating these compounds may bind distant from the active site and may have an allosteric impact on pNP-TMP hydrolysis.

Table 3: Nitro analog activity data

Analog	FS-3 Inhibition (%)	FS-3 IC ₅₀ (μM)	pNP-TMP Inhibition (%)	pNP-TMP IC ₅₀ (μM)
1	47.1 ± 4.4	---	18.7 ± 0.9	---
2	25.6 ± 5.1	---	-1.8 ± 4.5	---
3	50.0 ± 2.7	11.4 ± 4.7	38.2 ± 2.3	---
4	42.7 ± 1.2	---	24.9 ± 1.0	---
5	39.5 ± 2.5	---	18.2 ± 3.8	---
6	49.2 ± 1.5	---	35.4 ± 1.6	---
7	59.1 ± 1.1	8.2 ± 0.9	38.0 ± 1.3	---
8	42.4 ± 4.4	---	19.9 ± 3.7	---
9	72.6 ± 1.5	4.7 ± 1.1	44.7 ± 2.0	11.3 ± 0.3
10	69.8 ± 0.8	4.1 ± 0.0	37.1 ± 1.3	---
11	77.7 ± 1.0	3.0 ± 0.1	46.8 ± 1.0	11.4 ± 0.3
12	41.9 ± 1.7	---	4.9 ± 0.8	---
13	15.2 ± 5.5	---	-14.7 ± 2.9	---
14	56.4 ± 1.0	6.7 ± 0.6	7.7 ± 3.3	---
15	62.4 ± 1.7	5.8 ± 0.3	17.1 ± 1.4	---
16	59.8 ± 2.5	6.2 ± 1.6	13.3 ± 2.4	---
17	61.9 ± 1.1	4.6 ± 0.1	14.3 ± 2.9	---
18	62.6 ± 2.1	5.4 ± 0.1	23.8 ± 2.9	---
19	48.1 ± 3.2	---	8.9 ± 1.2	---
20	60.4 ± 0.8	4.2 ± 0.1	30.3 ± 2.0	---
21	73.2 ± 1.7	3.4 ± 0.3	7.3 ± 2.0	---
22	84.3 ± 0.8	0.67 ± 0.1	52.1 ± 0.9	8.9 ± 0.4
23	46.1 ± 0.3	---	-11.9 ± 1.3	---
24	35.2 ± 1.4	---	-22.7 ± 4.2	---
25	54.6 ± 6.6	9.2 ± 0.6	-3.7 ± 2.6	---
26	70.6 ± 1.7	4.6 ± 0.6	15.6 ± 1.2	---
27	63.0 ± 9.2	6.6 ± 0.6	-14.1 ± 2.3	---
28	63.3 ± 0.4	4.9 ± 0.2	4.1 ± 1.2	---
29	76.0 ± 0.7	2.2 ± 0.2	19.9 ± 1.3	---
30	65.4 ± 3.2	6.4 ± 0.5	-12.1 ± 1.2	---
31	50.2 ± 1.8	8.1 ± 0.6	26.6 ± 2.0	---
32	51.8 ± 5.8	3.7 ± 0.4	-14.9 ± 4.7	---
33	63.0 ± 0.4	2.3 ± 0.1	20.4 ± 0.6	---

Table 4: Carboxy analog activity data

Analog	FS-3 Inhibition (%)	FS-3 IC ₅₀ (μM)	pNP-TMP Inhibition (%)	pNP-TMP IC ₅₀ (μM)
34	12.1 ± 8.3	---	1.7 ± 2.0	---
35	-7.6 ± 4.2	---	-5.7 ± 2.7	---
36	17.3 ± 4.5	---	4.6 ± 2.2	---
37	24.4 ± 3.9	---	-3.3 ± 2.4	---
38	-5.5 ± 8.6	---	-4.8 ± 4.9	---
39	41.3 ± 6.9	---	-3.5 ± 3.7	---
40	74.5 ± 3.2	6.0 ± 1.8	32.1 ± 1.6	---
41	11.5 ± 11.0	---	-7.2 ± 5.4	---
42	89.1 ± 1.1	1.8 ± 0.4	43.7 ± 3.2	---
43	27.6 ± 1.5	---	-7.9 ± 2.8	---
44	59.8 ± 4.8	2.2 ± 0.3	23.4 ± 4.5	---
45	42.0 ± 4.2	---	11.5 ± 3.2	---
46	10.8 ± 1.7	---	10.9 ± 4.1	---
47	51.5 ± 1.1	8.1 ± 1.2	23.7 ± 2.5	---
48	80.0 ± 0.9	2.6 ± 0.2	37.1 ± 0.5	---
49	55.2 ± 4.7	8.9 ± 1.3	30.2 ± 6.8	---
50	67.1 ± 3.0	2.2 ± 0.6	37.9 ± 3.1	---
51	79.8 ± 2.6	3.7 ± 0.2	56.6 ± 1.5	6.2 ± 0.5
52	58.1 ± 1.4	4.6 ± 0.4	41.1 ± 9.3	---
53	65.0 ± 4.7	7.5 ± 0.4	31.6 ± 3.5	---
54	76.1 ± 0.7	2.1 ± 0.1	29.5 ± 1.9	---
55	54.6 ± 1.9	7.3 ± 0.1	56.2 ± 3.4	5.2 ± 0.1
56	27.6 ± 2.5	---	7.3 ± 3.9	---
57	6.1 ± 4.0	---	-12.0 ± 6.0	---
58	10.9 ± 1.8	---	-0.8 ± 2.1	---
59	56.0 ± 0.8	6.5 ± 0.8	6.8 ± 2.3	---
60	32.3 ± 1.2	---	-36.5 ± 6.3	---
61	43.0 ± 7.1	---	-11.4 ± 2.6	---
62	50.8 ± 0.8	4.1 ± 0.2	19.6 ± 4.3	---
63	0.9 ± 2.1	---	-6.5 ± 0.6	---
64	46.0 ± 3.1	---	7.9 ± 4.2	---
65	23.9 ± 4.1	---	9.7 ± 2.2	---
66	46.7 ± 3.7	---	27.8 ± 5.4	---

Specificity for ATX was determined by testing the twelve inhibitors more potent than the lead (figure 5) against NPP6 and NPP7, the only other NPP family members that are known to hydrolyze the common substrate LPC. Neither NPP6 nor NPP7 contains a hydrophobic pocket analogous to that of ATX. As such, we anticipated no effect on NPP6 and NPP7 activity. Indeed, none of these compounds showed inhibition greater than 16% at 10 μM for either enzyme (supplemental figure 5). Additionally, **22** showed little to no agonist nor antagonist activity with LPA₁₋₅ alone (data not shown) but it did show modest potentiation of LPA effects at LPA₂ (supplemental figure 6). These data suggest some level of specificity for ATX over related lipid phosphodiesterases and no effect at know LPA GPCR.

Figure 5: Twelve analogs of GRI392104 with improved potency.

4. Discussion

ATX has lysophospholipase D activity[2,3] leading to specific GPCR activation by its product LPA. The ATX-LPA axis has received interest as a drug target[44,45] due to its role in human disease. Initial headway toward ATX inhibition began with non-specific metal chelators[46], which inhibited ATX activity when applied in millimolar concentrations and which have numerous off target effects. Once LPA and S1P, both bioactive lipid products of ATX hydrolysis, were identified as feedback inhibitors of ATX[47], research shifted into inhibitory properties of LPA/S1P analogs.[48-51] Although, some of these compounds had promising *in*

vitro potencies, lipids are unlikely to be orally bioavailable drug candidates according to Lipinski's rules.[32] Efforts to generate better drug candidate ATX inhibitors resulted in identification of non-lipid inhibitors.[36,51-53] The first small molecule, non-lipid inhibitor was reported in 2008.[52] Other diverse small molecules were discovered in efforts to improve potency.[23,36,51,53-56] Because protein structure and function are closely related, it is important to understand how molecules bind to ATX in an effort to better design/identify potent inhibitors. The recently published crystal structures of ATX, enzyme inhibition kinetics, and molecular docking studies show distinct binding regions for small molecule ATX inhibitors.[26-30] Small molecule, non-lipid inhibitors are divided between their interactions with either the polar active site[30,53-55] and the distant hydrophobic domain.[28-30,53,57]

In the present study, a structure-based pharmacophore was generated for the hydrophobic region of ATX in order to discover novel inhibitor scaffolds which are likely to show selectivity for ATX over other NPP enzymes (NPP6 and NPP7) that lack the hydrophobic pocket. These molecules would also be smaller and more rigid than the relatively large and flexible LPA which would likely prevent their interaction with known LPA GPCR. After searching a large database of nearly 400,000 compounds, seventy-two candidate inhibitors were tested with four showing inhibition of ATX (table 2). The most potent and synthetically tractable inhibitor, GRI 392104 (IC₅₀ 4 μ M) was used as a scaffold to explore a structure-activity relationship. Sixty-six analogs (table 1) were synthesized and analyzed for ATX inhibition.

This study used two synthetic substrates to differentiate binding modes without the need to solve crystallographic structures as both Albers *et al.* and Kawaguchi *et al.* did, which follows a precedent set by Hoeglund *et al.*, Saunders *et al.*, and Fells *et al.*[28,43,55,58,59] FS-3 (Echelon)[41] is a large substrate with increased fluorescence after hydrolysis by ATX. FS-3 should occupy the entire binding pocket occupied by LPC, the endogenous substrate, stretching from the polar active site down into the hydrophobic pocket. Another substrate, 4-nitrophenyl thymidine-5'-monophosphate (pNP-TMP, Sigma) is synthetic nucleotide which can be hydrolyzed to release 4-nitrophenolate to absorb light at 405 nm. This smaller substrate, pNP-TMP, should only reside in the polar active site and not extend into the hydrophobic region. Inhibitors that block FS-3 hydrolysis but not the phosphodiesterase activity of ATX toward pNP-TMP suggests interactions distant from the polar active site deeper in the hydrophobic region of the catalytic domain. However, some analogs may still have positive or negative allosteric effects on pNP-TMP hydrolysis, resulting in either an increase or decrease in ATX activity on that substrate (for instance, **32** inhibits FS-3 hydrolysis 52% but increases ATX-catalyzed hydrolysis of pNP-TMP while **22** inhibits ATX activity for both FS-3 and pNP-TMP to varying degrees).

Thirty-six of the analogs synthesized showed ATX inhibition greater than 50% at 10 μ M with FS-3, twelve of which had improved potency over the lead (figure 5). Of these, **22** was sub-micromolar (IC₅₀ 670 nM). The 3,5-dichlorophenoxy ring of **22** is reminiscent of the 3,5-dichlorophenylthiourea ring in Hoeglund 5 (IC₅₀ 1.6 μ M, figure 6), which was previously shown to be a competitive inhibitor.[53] Like **22**, PF-8380 (IC₅₀ 2.8 nM, figure 6) also has a 3,5-

dichloro substituted aromatic ring, but is attached through a methyl 1-piperazinecarboxylate.[36] Although **22** is similar to both Hoeglund 5 and PF-8380, neither of those scaffolds have a substituted phenoxy ring, which may lend to the activity of **22**. A structure-activity relationship study of PF-8380 also showed the importance of a 3,5-dichloroaromatic ring, but this study also did not use a substituted phenoxy ring.[60] Only one of the analogs with the nitro group ortho to the piperazine ring (**11**, IC₅₀ 3 μ M) and two ortho carboxy analogs (**42**, IC₅₀ 1.8 μ M and **44**, IC₅₀ 2.2 μ M) showed improved potency over the lead, indicating ortho substitution on the phenyl piperazine ring may not lead to further improvement. A similar trend was seen with boronic acid substitution on an aromatic ring by Albers *et al.* (figure 6), where Albers 74 (IC₅₀ > 5 μ M) was far less potent than Albers 72 or 73 (IC₅₀ 28 nM and 5.7 nM, respectively) when monitoring choline release from the natural substrate, LPC.[54]

Inhibitor	R ₁	R ₂	R ₃	IC ₅₀ (μ M)	Substrate
Albers 72	H	B(OH) ₂	H	0.028	LPC
Albers 73	H	H	B(OH) ₂	0.0057	LPC
Albers 74	B(OH) ₂	H	H	> 5	LPC
Hoeglund 11	H	CF ₃	H	900	FS-3
Hoeglund 12	CF ₃	H	H	N/A	FS-3
Hoeglund 31	H	H	CF ₃	1.5	FS-3

Figure 6: Previously discovered non-lipid ATX inhibitors.

In general, using a shorter linker than the one in GRI392104 may help limit off-target effects by reducing flexibility[61] and does not seem to have detrimental effects on ATX inhibition (**32**,

IC₅₀ 3.7 μ M is quite similar to GRI392104, IC₅₀ 4 μ M). It is also of note that only two of the fluoro-containing compounds (**42** and **50**, IC₅₀ 2.2 μ M) improved potency over the lead. Of the fluoro-containing analogs, both **42** and **50** contain trifluoromethyl-substituted phenoxy rings in the para and meta positions, respectively. Ortho substitution on the phenoxy ring decreased potency (tables 3 and 4), following a similar pattern noticed by both Albers *et al.* and Hoeglund *et al.* during optimization of disparate lead compounds (figure 7).[54,56] All but five of the twelve compounds with improved activity over GRI 392104 had dichloro substitution on the phenoxy ring. This may suggest that, like PF8380 and Hoeglund 5, having a dichloro substitution may improve potency, as also seen during structure-activity relationship studies of PF8380.[60] Across the 66 compounds tested, minor changes in structure caused vast differences in biological activity. These drastic changes are activity cliffs, as described by both Dimova *et al.* and Hu *et al.*[62,63] Figure 7 shows a generic structure of these compounds, highlighting similarities in PF8380 and Hoeglund 5. Based on results presented here and by extrapolation to related work of others we propose that optimization of the polar head group of this scaffold can serve as a targeting handle whereas changes to the non-polar aromatic moiety can be implemented to improve pharmacokinetics and biological stability – although the 3,5-dichloro moiety is preferred.

Figure 7: Common Structure of **1-66**, PF8380, and Hoeglund 5. *R* is *O* in **1-66** and PF8380 but *S* in Hoeglund 5.

Although ATX activity is reduced by these inhibitors, they are unlikely to be used as the lone treatment against ATX-associated diseases without further study in more complex systems. These compounds may be useful by causing inhibitor-mediated decreases in ATX activity, which would sensitize tumor cells to radiotherapy[64] and apoptosis caused by treatments such as Taxol.[65] It is important to explore several different scaffolds when developing targeted inhibitors because of the high attrition rate moving hit compounds through the development pipeline to approved drugs.[66,67] By diversifying the number of ATX inhibitors, there is greater hope of successfully treating ATX-related diseases.

5. Conclusion

Here we describe a novel ATX inhibitor scaffold that was synthetically modified to improve potency into the sub-micromolar range. This study also demonstrates that shortening the carbon

linker on this scaffold has no apparent detrimental effect and, in fact, may be of more use for further development and modification to produce an orally bioavailable drug. While flexible compounds may be useful for binding to ATX, they may also adopt additional conformations leading to off-target effects. Out of the sixty-six new analogs synthesized, twelve showed improved potency with one of those improving potency into the sub-micromolar range (**22**, IC₅₀ 670 nM versus GRI 392104, IC₅₀ 4 μ M). Future endeavors are ongoing to further optimize this lead and to characterize both pharmacokinetic and pharmacodynamic effects.

Acknowledgements

This work was supported by the FedEx Institute of Technology at The University of Memphis (to DLB and ALP), NSF REU CHE 1156738 (to ALP in support of RSS), and NIH CA921060 (to GT). This material is based upon work supported by the National Science Foundation under Grant No. CHE-1531466. Thanks also to the Chemical Computing Group, Montreal, Canada for the Molecular Operating Environment (MOE). We thank Dr. Roberto de la Salud Bea from Rhodes College for synthesis advice. We also thank Daniel Hoagland and Dr. Richard Lee from St. Jude Children's Research Hospital for purity testing of **22**.

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