Structural Basis for Inhibition of Human Autotaxin by Four Potent Compounds with Distinct Modes of Binding

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ABSTRACT

Autotaxin (ATX) is a secreted enzyme that hydrolyzes lysophosphatidylcholine to lysophosphatic acid (LPA). LPA is a bioactive phospholipid that regulates diverse biological processes, including cell proliferation, migration, and survival/apoptosis, through the activation of a family of G protein–coupled receptors. The ATX-LPA pathway has been implicated in many pathologic conditions, including cancer, fibrosis, inflammation, cholestatic pruritus, and pain. Therefore, ATX inhibitors represent an attractive strategy for the development of therapeutics to treat a variety of diseases. Mouse and rat ATX have been crystallized previously with LPA or small-molecule inhibitors bound. Here, we present the crystal structures of human ATX in complex with four previously unpublished, structurally distinct ATX inhibitors. We demonstrate that the mechanism of inhibition of each compound reflects its unique interactions with human ATX. Our studies may provide a basis for the rational design of novel ATX inhibitors.

Introduction

Autotaxin (ATX, also known as ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2)) is an ~100-kDa secreted glycoprotein that was originally identified as a tumor cell autocrine motility factor and later shown to be responsible for the lysophospholipase D (lysoPLD) activity in human serum that hydrolyzes lysophosphatidylcholine (LPC) to generate lysosphatic acid (LPA) (Stracke et al., 1992; Tokumura et al., 2002; Umezu-Goto et al., 2002; Gijsbers et al., 2003). ATX homozygote knockout mice are embryonic lethal due to abnormal blood vessel development, but ATX heterozygotes are phenotypically normal with approximately 50% circulating LPA compared with wild-type mice (Tanaka et al., 2006; van Meeteren et al., 2006). LPA stimulates multiple cell signaling pathways through the activation of at least six cell surface G protein–coupled receptors, and the ATX-LPA pathway has been implicated in a number of physiologic and pathologic processes (Yung et al., 2014). Serum LPA and ATX lysoPLD activity are elevated in many disease settings, including renal cancer (Su et al., 2013), glioblastoma (Kishi et al., 2006), follicular lymphoma (Masuda et al., 2008), liver cirrhosis (Kondo et al., 2014), primary biliary cirrhosis (Kremer et al., 2010), and atopnic dermatitis (Shimizu et al., 2014), so it is of great interest to the pharmaceutical and medical community to delineate the therapeutic benefit of LPA receptor antagonism or ATX enzyme inhibition.

ATX is most structurally related to ENPP1 and ENPP3, two other members of the seven-member ENPP family; however, within this family, only ATX exhibits lysoPLD activity (Stefan et al., 2005). ATX retains pyrophosphatase (PPase)/phosphodiesterase (PDE) activities (Clair et al., 1997) and can cleave artificial substrates such as FS-3 and bis(4-nitrophenyl)phosphate (BNPP) (Ferguson et al., 2006). However, it is unclear if the residual PPase/PDE activities of ATX have any physiologic or pathologic consequences in vivo. There are four ATX isoforms (ATXa, β, γ, and δ) that are derived from differential splicing, and ATXδ and ATXγ are the major stable circulating isoforms (Giganti et al., 2008; Hashimoto et al., 2012). ATX has two somatomedin B (SMB)–like binding domains at the amino terminus which are cysteine-rich domains that are known to mediate protein-protein interactions. The SMB2 domain binds to β1 and β3 integrins (Fulkerson et al., 2012).

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ABBREVIATIONS: AA, arachidonic acid; ATCC, American Type Culture Collection; ATX, autotaxin; BNPP, bis(4-nitrophenyl)phosphate; ENPP, ectonucleotide pyrophosphatase/phosphodiesterase; HA155, (Z)-(4-((4-((3-(4-fluorobenzyl)-2,4-dioxothiazolidin-5-ylidene)methyl)phenoxy)methyl)-1H-indolin-2(3H)-yl)thio)-2-fluorobenzoic acid; HBM, honeybee melittin; HEK, human embryonic kidney; ID, identifier; LPA, lysophosphatic acid; LPC, lysophosphatidylcholine; lysoPLD, lysophospholipase D; PAT-347, 3-((6-chloro-7-fluoro-2-methyl-1-(2-oxo-2-(spiro[cyclopropane-1,3]-piperazin-1-ylidene)methyl)phenoxy)methyl)-1H-indolin-2(3H)-yl)propionic acid; PAT-352, (S)-(3)-(6-(4-fluorobenzyl)-1,3-dioxo-5,6,11,11a-tetrahydro-1H-imidazo[1′,5′:1,5]pyridrid[3,4-b]indol-2(3H)-yl)propanoic acid; PAT-484, 6-(4-fluorobenzyl)-5,6,11,11a-tetrahydro-1H-imidazo[1′,5′:1,5]pyridrid[3,4-b]indole-1,3(2H)-dione; PDB, Protein Data Bank; PDE, phosphodiesterase; PF-8380, 3,5-dichlorobenzyl 4-[(3-oxo-3-[2-oxo-2,3-dihydrobenzo[|oxazol-6-yl]propyl]piperazine-1-carboxylate; PPase, pyrophosphatase; SMB, somatomedin B.
2011), which may provide a mechanism for generating LPA in close proximity to its cognate receptors (Hausmann et al., 2011). The SMB domains are followed by a central catalytic domain which binds two zinc ions and contains an active site threonine and an N-glycan, all of which are critical for the lysolPLD and PPase/PDE activities of ATX (Gijbers et al., 2003; Jansen et al., 2007). The C terminus contains the nucleaase-like domain, which is catalytically inert. The X-ray crystal structures of mouse [Protein Data Bank (PDB) identifier (ID) 3NKM] (Nishimasu et al., 2011) and rat (PDB ID 2XR9) (Hausmann et al., 2011) ATX have been previously described and show that the SMB domains and the nuclease domain sandwich the catalytic domain and stabilize it. From these crystal structures, it is clear that the catalytic domain includes a hydrophobic lipid-binding pocket; however, a second hydrophobic channel was identified and was suggested to function as either an entrance channel for the LPC substrates or an exit channel for the delivery of LPA to its cognate G protein–coupled receptors (PDB ID 3NKN) (Nishimasu et al., 2011). Here, we describe the crystal structures of human ATX in complex with four potent, previously unpublished inhibitors and correlate the mode of inhibition of these inhibitors with each of their unique ATX binding characteristics.

Materials and Methods

Human ATX Cloning, Mutagenesis, Recombinant Protein Expression, and Purification for Crystallography

The ATX cDNA was obtained from Open Biosystems (part of GE Healthcare Bio-Sciences, Pittsburgh, PA). ATX (amino acids 36–863) was polymerase chain reaction amplified with a C-terminal octa-histidine tag extension. The amplified ATX-C8His gene was ligated into a pFastBac (Life Technologies, Carlsbad, CA) donor plasmid previously generated with an N-terminal honeybee melittin (HBM) signal peptide sequence. The resulting pFastBac-HBM-ATX-C8His construct was then used to generate DNA mutations that would result in the incorporation of alanine residues at amino acid positions 54 and 411 instead of the normally occurring asparagine residues. The DNA mutations were introduced using the QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacture’s instructions. The following primers were used to introduce the mutations: N54A mutation forward primer 5'-GACTCCCTGGACCCATCTCGGATCTCTG and reverse primer 5'-CAAGATCCCCAGATGGCCCTGGGAGCAGCTC, and N411A forward primer 5'-CCCCAACGCGATTGCGCCCTCTGCTGTCGTTAAAAACC-CC and reverse primer 5'-GGTTTTACTACGTTAGAGCAGCGCAATAATGGCCTTGGG. The resulting pFastBac-HBM-ATX double mutant (N54A/N411A)-C8His construct was then inserted into DH10Bac cells (Life Technologies), and bacmid DNA was isolated and used to transfect Sf21 cells. Virus was amplified and used to generate baculovirus-infected insect cells stocks. Amplified virus or baculovirus-infected insect cells stocks were used to express mature secreted ATX. Protein expression in media was analyzed by immunoblotting using rabbit anti-ATX polyclonal antibody (Cayman Chemical, Ann Arbor, MI). After 3 days, in media was analyzed by immunoblotting using rabbit anti-ATX polyclonal antibody (Cayman Chemical, Ann Arbor, MI). After 3 days, 6 l of media was collected and filtered using a glass prefilter and 5,6,11,11a-tetrahydro-1H-imidazo [1,6-b]pyrido[3,4-b]indol-2(3H)-yl)propanoic acid (PAT-352) were determined as described for ATX/PAT-352. The electron density for the secondary molecules was used as the search model. The solution was refined using rigid body refinement in REFMAC (Vagin et al., 2004). After rigid body refinement, rounds of manual rebuilding were followed by cycles of torsion-angle dynamics, positional refinement, and individual B-factor refinement using COOT (http://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/) (Emsley and Cowtan, 2004) and REFMAC (http://www2.mrc-lmb.cam.ac.uk/groups/murshudov/). This was all performed in the absence of the inhibitor. After placement of the solvent molecules, the inhibitor was modeled and refined.

Structure Determination

All refinement statistics are listed Table 2.

General procedure: Refinement of ATX_Inhibitor Structures.

The structure of an ATX/inhibitor complex was determined by molecular replacement techniques as implemented in PHASER (http://www.phaser.cimr.cam.ac.uk/index.php/Phaser_Crystallographic_Software) (McCoy et al., 2007). The solution was refined using rigid body refinement in REFMAC (Vagin et al., 2004). After rigid body refinement, rounds of manual rebuilding were followed by cycles of torsion-angle dynamics, positional refinement, and individual B-factor refinement using COOT (http://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/) (Emsley and Cowtan, 2004) and REFMAC (http://www2.mrc-lmb.cam.ac.uk/groups/murshudov/). This was all performed in the absence of the inhibitor. After placement of the solvent molecules, the inhibitor was modeled and refined.

ATX/PAT-078. The mouse ATX crystal structure (PDB ID: 3WAY) was used as the search model. The solution was refined, and inspection of the initial electron density maps showed unambiguous density for ATX/PAT-078 in the LPA binding site.

ATX/PAT-352. The ATX/PAT-078 crystal structure without any of the secondary molecules was used as the search model. The solution was refined and the initial electron density maps showed unambiguous density for two molecules of (S)-3-(6-(4-fluorobenzyl)-1,3-dioxo-5,6,11,11a-tetrahydro-1H-imidazo[1’5,1’6:1,6]pyrido[3,4-b][1,2]furan-3(4H)-yl)propanoic acid (PAT-352)—one partially in the LPA binding site as well as a secondary allosteric site. Further refinement was performed as described for ATX/PAT-078.

ATX/PAT-347 and ATX/PAT-494. The structures of both ATX/PAT-347 and ATX/PAT-494 were determined as described for ATX/PAT-352. The electron density for ATX/PAT-347 and ATX/PAT-494 are also shown in Supplemental Fig. 1, and the

Crystallization and Data Collection.

General crystallization protocol. Complex ATX/inhibitor was made by adding 5 mM inhibitor to 11 mg/ml double mutant ATX. Crystals of ATX/inhibitor were grown at room temperature by sitting-drop vapor diffusion against a reservoir containing 10–20% polyethylene glycol 3500 (mother liquor) and various salts. Crystals were quickly transferred into a cryoprotectant containing the mother liquid supplemented with 20% glycerol and flash frozen in liquid nitrogen prior to data collection. All data were collected at 160°C at the LS-CAT (21-ID) beamline at Argonne National Laboratory (Lemont, IL). Data were integrated, scaled, and merged using HKL2000 (HKL Research, Charlottesville, VA) (Otwinowski and Minor, 1997).
Protein Production for ATX Isoforms

Each of the four ATX isoforms was cloned by standard polymerase chain reaction techniques into the XbaI/BamHI site of pcDNA3.1(−) (ThermoFisher Scientific, Grand Island, NY), using the following primer pair: forward primer 5′-TCTAGACCATGGCGAAGGA-GAGCCCTGTTCC and reverse primer 5′-GGATCCCTAAATCCGGTCTCATATGTAC. ATXα and δ were cloned from human skeletal muscle cDNA, human ATXβ was cloned from human kidney cDNA, and human ATXγ was cloned from human brain cDNA. All cDNAs were verified by sequence analysis. For production of ATX protein, 293T/17 [human embryonic kidney-293T/17 (HEK-293T/17) [CRL-11268; American Type Culture Collection (ATCC), Manassas, VA] cells were transfected with each expression construct using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Cells were plated 1 day prior to transfection, and 7 hours after transfection, medium was removed and the cells washed once with phosphate-buffered saline before the addition of serum-free, phenol red–free medium. Cells were incubated for an additional 48 hours at 37°C and 5% CO2 before the conditioned medium was collected, centrifuged to remove cells and debris, and frozen at −80°C. Concentrated conditioned medium from Hep 3B2.1-7 [Hep 3B, Hep-3B, Hep3B] (ATCC HB-8064) cells was generated by culturing the cells in growth medium until ∼80% confluence. The cells were then washed once with phosphate-buffered saline before the addition of serum-free, phenol red–free growth medium. Cells were incubated for an additional 48 hours at 37°C and 5% CO2 before the conditioned medium was collected and centrifuged to remove cells and debris. The conditioned medium was concentrated 10–20× using 30K MWCO Centriprep centrifugal filters (Millipore, Billerica, MA) according to the manufacturer’s instructions.

LysoPLD Activity Assay

ATX lysoPLD activity was measured by the release of choline from the substrate, 14:0 LPC. In brief, conditioned medium from transiently transfected 293T/17 [HEK-293T/17 (ATCC CRL-11268) cells] or concentrated conditioned medium from Hep 3B2.1-7 [Hep 3B, Hep-3B, Hep3B] (ATCC HB-8064) cells was incubated with inhibitor (or LPA, AA, or vehicle control) in lysoPLD buffer [100 mM Tris (pH 9), 500 mM NaCl, 5 mM MgCl2, 5 mM CaCl2, and 0.05% Triton X-100] for 15 minutes at 37°C before the addition of 14:0 LPC to 100 μM. After an additional 1.5-hour incubation at 37°C, the liberated choline was detected by the equal volume of detection reagent (4.5 mM 4-aminoantipyrine, 2.7 mM N-ethyl-N-(2-hydroxy-3-sulfopropyl)toluidine, 20 units ml−1 horseradish peroxidase, and 3 units ml−1 choline oxidase in 50 mM Tris (pH 8), 4.5 mM MgCl2) and a further incubation for 10 minutes at room temperature. Color development was detected using a Molecular Devices SpectraMax Plus for measuring the absorbance at a wavelength of 555 nm. The concentration of choline was calculated from a standard curve generated using choline chloride. The ATX compounds were tested for inhibition of the choline oxidase and horseradish peroxidase enzymes using a choline standard curve and were shown to have no inhibition of these enzymes at concentrations that showed 100% inhibition in the lysoPLD assay (1 μM inhibitor; data not shown).

BNP Cleavage Assay

The ability of ATX to cleave the nucleotide-like substrate BNPP was determined by measuring the generation of the yellow product, p-nitrophenyl. In brief, conditioned medium from 293T/17 [HEK-293T/17 (ATCC CRL-11268) cells] transfected with human ATXβ was incubated with inhibitor (or LPA, AA, or vehicle control) in assay buffer [50 mM Tris (pH 8), 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 5 mM KCl] for 15 minutes at 37°C before the addition of BNPP to 1.5 mM. After an additional 1- to 3-hour incubation at 37°C, the liberated p-nitrophenyl was detected using a Molecular Devices SpectraMax Plus by measuring the absorbance at 405 nm.

FS-3 Cleavage Assay

The ability of ATX to cleave FS-3 was determined fluorometrically. In brief, conditioned medium from 293T/17 [HEK-293T/17 (ATCC CRL-11268) cells] transfected with human ATXβ was incubated with varying concentrations of 14:0 LPC in the absence or presence of inhibitor (0.5–4 times the IC50 value determined from concentration response curves) in lysoPLD buffer for 1.5 hours at 37°C before the addition of the color reagents for detection of released choline. The data were plotted and substrate/velocity curves generated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Km and Vmax values were generated in GraphPad Prism 6 from nonlinear regression analysis of the Michaelis-Menten data.

Western Blotting

ATX expression in conditioned media was evaluated by Western blotting using an affinity-purified polyclonal goat IgG against human ATX (catalog number AF5255; R&D Systems, Minneapolis, MN). In brief, conditioned medium from 293T/17 [HEK-293T/17 (ATCC CRL-11268) cells] transfected with ATXα, β, γ, or δ concentrated conditioned medium from Hep 3B2.1-7 [Hep 3B, Hep-3B, Hep3B] (ATCC HB-8064) cells was separated on Bolt 4–12% Bis-Tris protein gels (Life Technologies) and then transferred to nitrocellulose using the iBlot gel transfer device (Life Technologies). Blots were incubated with the anti-ATX antibody (1:250 dilution) for 2 hours at room temperature before incubation with an IRDye conjugated anti-goat secondary antibody and imaging with an Odyssey CLx (LI-COR Biosciences, Lincoln, NE).

Human Blood LysoPLD Assay

The lysoPLD activity of ATX in human blood was evaluated by measuring the generation of 20:4 LPA from endogenous LPC. Blood was collected from consenting human volunteers into heparin Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and used within 1 hour of draw. Prior to setting up the assay, an aliquot of blood was removed, centrifuged at 800 × g for 10 minutes at 4°C to isolate plasma, and the plasma was used for the determination of baseline 20:4 LPA concentrations. To assay for ATX activity, 200 μl of blood was incubated with inhibitor (or dimethylsulfoxide vehicle control) for 4 hours at 37°C. After the incubation, the blood was centrifuged as previously described to isolate plasma, and 40 μl of the plasma was
processed for the determination of 20:4 LPA concentrations by liquid chromatography–mass spectrometry. The plasma was processed by the addition of 5 volumes of ice-cold methanol containing 125 ng/ml 17:0 LPA as an internal standard and incubation at −20°C for 10 minutes before centrifuging at 4000 × g for 10 minutes at 4°C. One hundred fifty microliters of the supernatant was transferred to a 96-well plate and diluted with 100 μl of an organic solution (90:10:0.1 of water/acetonitrile/ammonium hydroxide) for analysis of 20:4 LPA concentrations by liquid chromatography–mass spectrometry. LPA 20:4 and the internal standard (LPA 17:0) were analyzed on a quadrupole mass spectrometer (4000QTrap AB Sciex, Framingham, MA) in the negative ion mode (electrospray ionization) by multiple reaction monitoring. The mobile phases contain 0.1% ammonium hydroxide in 90% water/10% acetonitrile (solvent A) and 0.1% ammonium hydroxide in 90% acetonitrile/10% water (solvent B). The flow rate was maintained at 0.8 ml/min, and the total run time was 3 minutes. Analytes were separated using a linear gradient as follows: 1) mobile phase was held for 0.5 minute at 10% B; 2) B was increased from 10 to 90% over the next 1 minute; 3) B was held constant for 0.5 minute at 90%; and 4) B was returned to the initial gradient conditions.

Results

The Structure and Inhibition of Human Autotaxin. A variant of full-length human ATX (residues 36–863, β-isoform) was generated in which the asparagine glycosylation sites at amino acid positions 54 and 411 were mutated to alanines (N54A and N411A) (Fig. 1A) (Hausmann et al., 2011). The two glycosylation sites were mutated to potentially enhance crystal formation. This double-deglycosylation mutant ATX was expressed in Sf21 insect cells, purified, and found to be appropriately reduced in molecular weight and with reduced catalytic activity compared with the wild-type protein (Supplemental Fig. 3, A and B) (Pradere et al., 2007). X-ray data from human ATX cocrySTALLized with four potent ATX inhibitors derived from internal research at PharmAkea (San Diego, CA) (Fig. 2A) were obtained at various resolutions by molecular replacement as described in the Methods. The data collection and crystal refinement details for each of the four structures are outlined in Table 1. The human ATX sequence is >90% identical to rodent sequences (Sievers et al., 2011), and the domain organization and overall structure show high homology to the previously crystallized mouse (PDB ID 3NKM) (Nishimatsu et al., 2011) and rat (PDB ID 2XR9) (Hausmann et al., 2011) ATX enzymes (Fig. 1B).

The potencies of two published ATX inhibitors, (Z)-4-[(3-(4-fluorobenzyl)-2,4-dioxothiazolidin-5-ylidene)methyl]phenoxy)methyl)phenyl)boronic acid (HA155) (Albers et al., 2010) and 3,5-dichlorobenzyl 4-(3-oxo-3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propyl)piperazine-1-carboxylate (PF-8380) (Gierse et al., 2010), and four previously unpublished ATX inhibitors (PAT-078, PAT-347, PAT-494, and PAT-352) (Fig. 2A) were determined against distinct enzymatic activities of ATX, including the lysopLD and PPase/PDE activities, by using diverse substrates, including LPC, FS-3, and BNPP (Fig. 2B). Additionally, the potencies of these inhibitors against the lysopLD activity of the four different ATX isoforms (α, β, γ, δ) were evaluated using conditioned medium from HEK-293 cells recombinantly expressing each isoform (Supplemental Fig. 4, A–E). Potencies against the lysopLD activity of endogenous ATX enzymes were evaluated using concentrated conditioned media from Hep3B human hepatocellular carcinoma cells and human blood. LysopLD activity assays were carried out using LPC with a 14-carbon length fully saturated acyl chain (C14:0) since this was shown by optimization studies to be a preferred substrate species in vitro (Giganti et al., 2008). All six compounds tested inhibited the lysopLD activity of human ATXβ maximally, albeit with IC50 values that ranged from 0.002 to 0.472 μM (Fig. 2C; Table 2), and each was equipotent at inhibiting the lysopLD activity of the four ATX isoforms (Supplemental Fig. 4, C–E; Supplemental Table 1). All six test compounds maximally inhibited lysopLD activity of endogenous ATX from human Hep 3B cells and human blood, with rank-order potencies similar to the recombinant ATX inhibition (Supplemental Fig. 5, A and B; Supplemental Table 1). Interestingly, the six compounds varied in their ability to inhibit cleavage of the artificial substrates, BNPP and FS-3. HA155 and PF-8380 showed maximal 100% inhibition of BNPP cleavage with average IC50 values of 0.007 and 0.001 μM, respectively (Fig. 2D; Table 2), whereas three of the PAT compounds (PAT-347, -352, and -494) inhibited BNPP cleavage at only ~50–75% of maximum, but with similar IC50 values to HA155 and PF-8380 (0.003–0.006 μM) (Fig. 2D; Table 2). PAT-078 was a very poor inhibitor of BNPP hydrolysis, showing a maximum inhibition of only ~15% (Fig. 2D; Table 2). HA155, PF-8380, and two of the PAT compounds (PAT-078 and PAT-352) showed potent, approximately maximal inhibition of FS-3 cleavage with average IC50 values ranging from 0.0004 to 0.011 μM (Fig. 2E; Table 2); however, PAT-347 and PAT-494 were unable to inhibit the ATX-mediated cleavage of FS-3 (Fig. 2E; Table 2).

The mode of enzyme inhibition for lysopLD activity was evaluated for each PAT compound by generating substrate/velocity curves in the presence of varying concentrations of inhibitor and determining the effects on Km and Vmax (Fig. 3). Increasing concentrations of PAT-078 resulted in a linear increase in Km, very little change in Vmax, and a large α value (α = 52), a profile characteristic of a competitive inhibitor.
Increasing concentrations of PAT-347, PAT-494, or PAT-352 resulted in curvilinear increases in $K_m$ and curvilinear decreases in $V_{max}$ which are consistent with a noncompetitive mode of inhibition. PAT-347 had an $\alpha$ value close to 1 (Fig. 3, C and D), consistent with a true noncompetitive mode of inhibition, whereas PAT-352 and PAT-494 displayed higher $\alpha$ values which are more consistent with a mixed mode of inhibition (Fig. 3, E–H).

Crystal Structure of ATX Bound to the Competitive Inhibitor PAT-078. The crystal structure of human ATX bound to PAT-078 (PDB ID 4ZG6) showed that this inhibitor binds in the catalytic site and partially fills the substrate and
LPA/HA155 binding site (Fig. 4A) (Hausmann et al., 2011; Nishimasu et al., 2011). Two polar contacts exist between PAT-078 and ATX. The more important of the two contacts is the PAT-078 vinyl-nitrile-backbone coordination of Phe275 (3.1 Å). The other contact is a water-mediated interaction with the PAT-078 benzoic acid (3.2 Å) (Fig. 4A). Other key interactions include π-stacking between the vinyl indole and Tyr307 with the fluorophenyl group tightly packed into a pocket aligned with Leu217 and Ala218 (Fig. 4A). Leu214 complements the vinyl indole–Tyr307 interaction by packing

**TABLE 1**

Data collection and refinement statistics

Parentheses denote the highest resolution shell statistics.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LPC Substrate</th>
<th>BNP Substrate</th>
<th>FS-3 Substrate</th>
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</thead>
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<tr>
<td></td>
<td>Average IC50 (S.D.), n</td>
<td>Average Max Inhibition</td>
<td>Average IC50 (S.D.), n</td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>%</td>
<td>μM</td>
</tr>
<tr>
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<td>100</td>
<td>0.002 (0.002), 3</td>
</tr>
<tr>
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<td>0.002 (0.0003), 3</td>
<td>100</td>
<td>0.003 (0.002), 3</td>
</tr>
<tr>
<td>PAT-494</td>
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<td>100</td>
<td>0.006 (0.005), 3</td>
</tr>
<tr>
<td>PAT-352</td>
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<td>100</td>
<td>0.005 (0.001), 3</td>
</tr>
<tr>
<td>HA155</td>
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<td>100</td>
<td>0.007 (0.003), 3</td>
</tr>
<tr>
<td>PF-8380</td>
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<td>100</td>
<td>0.001 (0.000), 4</td>
</tr>
</tbody>
</table>

n, number of assays performed; NA, not active.
against the chloro-indole of PAT-078 (Fig. 4A). The carboxylic acid points toward the solvent and does not interact with the zinc. This is in contrast to the HA155 crystal structure in which the boronic acid interacts with both zinc atoms and the active site Thr210 (Thr209 in rat) (PDB ID 2XRG) (Hausmann et al., 2011) (Fig. 4B).

Crystal Structure of ATX Bound to the Noncompetitive Inhibitor PAT-347. The crystal structure of ATX bound to PAT-347 (PDB ID 4ZG7) showed that it cocrystallized with an endogenous C14:0 acyl chain LPA (14:0 LPA) (Fig. 4C). In the enzyme kinetic analysis, PAT-347 displayed a noncompetitive mode of inhibition with respect to the lysoPLD activity, and in the crystal structure, it binds in an allosteric site adjacent to the catalytic site (Fig. 4C). This noncompetitive site is the same as the previously reported putative secondary LPA binding site in the mouse ATX crystal structure, and has also been identified as a hydrophobic channel (Nishimasu et al., 2011). The 14:0 LPA binds in the catalytic site in a similar fashion to the 14:0 LPA bound in the mouse ATX crystal structure (PDB ID 3NKN) (Nishimasu et al., 2011). The PAT-347 indole forms a $\pi$-$\pi$ interaction with Phe275 (edge:face) with another $\pi$-$\pi$ interaction between His252 and the cyclopropyl-dihydroindole (face:face) (Fig. 4C). The PAT-347 benzoic acid makes polar contacts with a glycerol molecule added during the crystal cryoprotection process and is oriented toward the solvent boundary. Similarly, Phe250 introduces an edge on $\pi$-$\pi$ interaction with the benzoic acid (Fig. 4C). When the crystal structures of PAT-347/LPA are overlaid with PAT-078, competitive versus noncompetitive binding sites are easily distinguishable (Fig. 4D).

Fig. 3. Enzyme kinetics in the presence of inhibitors. (A, C, E, and G) Substrate/velocity curves and $\alpha$ values for ATX lysoPLD activity in the presence of varying concentrations (0.5–4 × the IC$_{50}$ value) of the ATX inhibitors (ATXi) PAT-078 (A), PAT-347 (C), PAT-494 (E), and PAT-352 (G). (B, D, F, and H) Plots of $K_m$ and $V_{max}$ values versus inhibitor concentration for PAT-078 (B), PAT-347 (D), PAT-494 (F), and PAT-352 (H). The $K_m$ and $V_{max}$ values were derived from the substrate/velocity curves shown in (A), (C), (E), and (G).
fluorophenyl group in a distant edge-face interaction. The consisting of Ser170, Leu217, and Phe274 surrounds the Phe211, Leu214, and Tyr215 (Fig. 5A). A second pocket tetracyclic core of PAT-494 is aligned in a pocket formed by the racemic compound; data not shown). The phenyl ring of the potent S-enantiomer (approximately 2-fold more potent than the X-ray structure that the enzyme has selected the more used in the crystallization process; however, it is clear from the Sf21 insect fatty-acid pools. Racemic PAT-494 was formation process, so the enzyme must have selected this molecule (Supplemental Fig. 6, B and C; data not shown). AA was not added during the crystallization process, so the enzyme has selected the more crystal structure (yellow) shows no overlap between the two sites. 4ZG6 represents the PDB deposition code for the ATX/PAT-347 crystal structure. 4ZGA represents the PDB deposition code for the ATX/PAT-078 crystal structure. 4ZG7 represents the PDB deposition code for the ATX/PAT-494 crystal structure. (Fig. 5C). A surface representation of both the LPA binding site identified in the PAT-347 crystal structure (orange) and the arachidonic acid binding site identified in the PAT-494 crystal structure (yellow) shows no overlap between the two sites. 4ZG6 represents the PDB deposition code for the ATX/PAT-078 crystal structure. 4ZGA represents the PDB deposition code for the ATX/PAT-494 crystal structure. 4ZG7 represents the PDB deposition code for the ATX/PAT-347 crystal structure.

Although LPA has been published to inhibit ATX activity (van Meeteren et al., 2005), we tested three different species of LPA (14:0, 18:1, or 20:4) and found that none of these LPA molecules has any effect on the lysoplD activity of ATX (Supplemental Fig. 6A; data not shown). However, all three species of LPA inhibited the ATX-mediated cleavage of BNPP and FS-3 (Supplemental Fig. 6, B and C; data not shown).

Crystal Structure of ATX Bound to the Mixed-Mode Inhibitor PAT-494. Crystallization with PAT-494 identified one molecule of PAT-494, and unexpectedly, one molecule of AA each bound in a distinct site outside the catalytic site (PDB ID 4ZGA) (Fig. 5A). AA was not added during the crystallization process, so the enzyme has selected this molecule from the Sf21 insect fatty-acid pools. Racemic PAT-494 was used in the crystallization process; however, it is clear from the X-ray structure that the enzyme has selected the more potent S-enantiomer (approximately 2-fold more potent than the racemic compound; data not shown). The phenyl ring of the tetracyclic core of PAT-494 is aligned in a pocket formed by Phe211, Leu214, and Tyr215 (Fig. 5A). A second pocket consisting of Ser170, Leu217, and Phe274 surrounds the fluorophenyl group in a distant edge-face interaction. The hydantoin of PAT-494 \( \pi \)-stacks with Tyr307 and one of the carboxyls makes an H-bond with the Phe275 amide, analogous to the PAT-078 cyano-amide bond (Fig. 5A). An overlay of the competitive inhibitor PAT-078 and the mixed-mode inhibitor PAT-494 (with AA cocrystallized) clearly shows that these two compounds occupy distinct but partially overlapping binding sites (Fig. 5B). Additionally, this overlay shows that the carboxylic acid of PAT-078 occupies similar space as the acid in AA, which would preclude AA from binding with PAT-078 (Fig. 5B). An overlay of the noncompetitive inhibitor PAT-347 and the mixed-mode inhibitor PAT-494 (with AA cocrystallized) shows that there is considerable overlap between the arachidonic acid binding site and the PAT-347 allosteric binding site within the hydrophobic channel (Fig. 5C). A surface representation of both the LPA binding site identified in the PAT-347 crystal and the AA binding site identified in the PAT-494 crystal clearly shows that these are two distinct binding regions (Fig. 5D). Although AA clearly binds to the enzyme in the PAT-494 crystal, when incubated with the enzyme alone, it is unable to inhibit the lysoplD activity of ATX (Supplemental Fig. 7A) or inhibit the cleavage of BNPP (Supplemental Fig. 7B) at concentrations up to 100 \( \mu \)M. However, AA does inhibit the ATX-mediated cleavage of FS-3 with an average IC\(_{50}\) value of 4.5 \( \mu \)M (Supplemental Fig. 7C).
Crystal Structure of ATX Bound to the Mixed-Mode Inhibitor PAT-352. Unexpectedly, the PAT-352 crystal structure showed a novel binding mode wherein two molecules of PAT-352 bound to the enzyme (PDB ID 4ZG9). One molecule of PAT-352 binds partially in the competitive site (site 1), analogous to PAT-494, and a second molecule binds in a secondary site adjacent to the competitive site (site 2) (Fig. 6A). The binding of PAT-352 in the secondary binding site is the result of a significant rearrangement in loop 240–255 (Fig. 6, B and C). The newly folded loop extends into the catalytic site where Arg247 hydrogen bonds to the PAT-352 carboxylic acid at the entrance to the hydrophobic pocket. As a result, the loop occludes the allosteric site occupied by PAT-347 and arachidonic acid (Fig. 6, B and C). Interestingly, the carboxylic acid of PAT-352 (site 1) binds in a region distinct from where the acids of PAT-078, PAT-347, and AA are located. In the PAT-352 secondary site, Trp254 from below, π-stacks with the indole ring of PAT-352 while the fluorophenyl ring is sandwiched between Arg440, Met233, and Phe242 from above (Fig. 6C). As shown in Fig. 6D, the overlay of all four crystal structures shows the distinct binding mode of each inhibitor as well as the cocrystallized 14:0 LPA and arachidonic acid molecules. The ATX structure shown as a semitransparent surface highlights the major structural domains, including the hydrophobic pocket which extends from the active site and the distinct allosteric/arachidonic acid binding site identified by the hydrophobic channel (Fig. 6E). The approximate volumes of 14:0 LPA bound in the hydrophobic pocket and arachidonic acid bound in the hydrophobic channel are depicted in Fig. 6F.

Discussion

The overall domain architecture of human ATX is very similar to that of the mouse (PDB ID 3NKM) (Nishimasu et al., 2011) and rat (PDB ID 2XR9) (Hausmann et al., 2011) enzymes. The binding of four potent, previously unpublished inhibitors synthesized at PharmAkeia was characterized in this study. These inhibitors represent three different structural classes (PAT-494 and PAT-352 are structurally related) and show some structural similarities. They all contain an indole ring with a phenyl containing indole N-substituent. Three of the inhibitors contain a carboxylic acid, whereas PAT-494 has an acidic hydantoin group. Additionally, all compounds have the acidic group extending from the C-3 position of the indole and potently inhibit the lysoPLD activity of ATX. However, despite these structural similarities, we noted significant differences in the mechanism of inhibition of the lysoPLD activity and differences in the abilities of these compounds to inhibit cleavage of the artificial substrates, FS-3 and BNPP.

PAT-078 is bound deep in the hydrophobic pocket of the competitive site, partially occluding the LPA and presumed LPC binding site. This binding would prevent substrate access, which is fully consistent with the competitive mode of inhibition. However, unlike the competitive inhibitor HA155, which coordinates to zinc (PDB ID 2XRG), PAT-078 does not extend far enough to interact with the active site zinc, and thus is unable to inhibit cleavage of the nucleotide-like BNPP substrate. In support of this hypothesis, Fells et al. (2013) have identified compounds that competitively inhibit cleavage of LPC-like substrates (LPC, FS-3), but also do not inhibit cleavage of nucleotide substrates. Molecular docking studies using these compounds identified a number of key interactions with ATX residues, including Leu214, Ala218, Phe275, and Tyr307, that correspond well with the ATX residues identified in this study (Fells et al., 2013). Further, the authors predicted that these compounds bind in the hydrophobic pocket away from the zinc ions in a similar manner to PAT-078. To test the hypothesis that competitive

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inhibitors require coordination with the zinc atoms to inhibit cleavage of nucleotide-like substrates, we rationally designed analogs of PAT-078 that would be expected to coordinate with the zinc atoms. These molecules remained competitive inhibitors of ATX, but now displayed 100% inhibition of BNPP cleavage with IC50 values of ∼0.01 μM (data not shown), thus supporting our hypothesis.

PAT-347 bound in an allosteric site in the enzyme is consistent with its noncompetitive mode of lysoPLD inhibition. Interestingly, in this crystal structure, LPA cocrystallized with PAT-347 and was shown to be bound in the active site/hydrophobic pocket. Since LPA was not added during the crystallization process, this LPA species was selected during the insect cell culture and carried throughout the protein purification process. Others have described the presence of 14:0 phospholipids and a Ca2+-independent phospholipase A (phospholipase A2) activity in SF9 cells (Yeh et al., 1997; Marheineke et al., 1998), so it is likely that the phospholipids can be converted to lysophospholipids within insect cells. The selection of the 14:0 LPA species is consistent with the identification of 14:0 LPC as a preferred ATX substrate in vitro (Giganti et al., 2008). The binding of LPA with the same acyl chain as the preferred LPC species suggests that the acyl chains of both likely fill similar space. Since LPA is bound in the hydrophobic pocket, any molecule that occupies similar space would prevent LPA from binding and cocrystallizing. PAT-078, PAT-494, and PAT-352 bind fully or partially in the hydrophobic pocket, so it is not surprising that these compounds did not cocrystallize with LPA. Although it is not entirely clear how allosteric compounds inhibit cleavage of LPC, one potential explanation is that allosteric binding does not affect cleavage of LPC, but blocks the generated LPA from exiting the enzyme through the hydrophobic channel, thereby trapping it and preventing binding of a second substrate molecule. Thus, one molecule of ATX bound to an allosteric inhibitor would bind and turn over one substrate molecule prior to being inhibited. Further biochemical studies with the allosteric inhibitors will be necessary to confirm this.

From the X-ray crystal structures, it is clear that PAT-494 binds orthogonally to PAT-078, and their binding sites only partially overlap. The hydantoin and vinyl-nitride portion of each molecule can both form a π-π interaction with Tyr307, and both make an H-bond with the Phe275 amide. These key interactions have been reported for other inhibitors cocrystallized with rodent ATX (Kawaguchi et al., 2013). However, unlike PAT-078, PAT-494 does not fully occupy the hydrophobic pocket, and this is consistent with its mixed mode of inhibition against the lysoPLD activity of human ATX. In the PAT-494 bound crystal structure, we unambiguously identified AA binding to the previously reported hydrophobic channel, which was hypothesized to be a putative second LPA binding site (PDB ID 3NKM) (Nishimasu et al., 2011). In the mouse ATX crystal structure, this site was found to be open to solvent and was suggested to interact with the plasma membrane and facilitate channeling of LPA to its cognate receptors (Nishimasu et al., 2011). Based on our results, the hydrophobic channel which binds AA also binds PAT-347, so binding of these two molecules is mutually exclusive. This is consistent with overlapping structural motifs on the two molecules. Both carboxylic acids are in the same region, and the terminal carbon chain of AA overlaps with the indole N-substituent of PAT-347. Although PAT-352 and PAT-494 have similar binding modes, AA does not cocrystallize with PAT-352. This is the result of the backbone rearrangement that occurs upon binding of PAT-352 to site 1, which shifts a protein loop such that it occupies the same hydrophobic channel where AA would bind, thus precluding AA binding. Similarly, AA does not cocrystallize with PAT-078, likely because the carboxylic acid of PAT-078 occupies the same space as the acid in AA, again precluding AA binding. Interestingly, unlike PAT-347, AA alone does not inhibit the lysoPLD activity of ATX, whereas it does inhibit the ATX-mediated cleavage of FS-3. AA binding to ATX in the crystal structure occurred in the presence of PAT-494, which may indicate a stabilization of the AA binding site by the inhibitor. Thus, the inability of AA to inhibit cleavage of LPC may be related to diminished AA binding to ATX in the absence of the PAT-494 ligand. The ability of AA alone to inhibit cleavage of FS-3 may reflect binding to a distinct site on the enzyme in the absence of PAT-494, such as the hydrophobic pocket.

Similar to PAT-494, the structurally related PAT-352 shows a mixed mode of inhibition against the lysoPLD activity of human ATX. It is therefore not surprising that PAT-352 (site 1) binds partially in the competitive site, similar to PAT-494, and makes very similar contacts with ATX with one major exception. PAT-352 interacts with Arg247 via coordination with both the carboxylic acid and a hydantoin carbonyl and induces a large-scale rearrangement of the 240–255 loop. As a result of this rearrangement, access to the hydrophobic pocket is restricted, and a second binding site for PAT-352 is revealed at the surface of the protein. PAT-352 and PAT-494 show similar inhibition modes and potencies with respect to the lysoPLD activity, and display similar potencies for inhibition of BNPP cleavage. However, maximum inhibition of BNPP cleavage by PAT-352 and PAT-494 is less than 100%, whereas the competitive ATX inhibitors, HA155 and PF-8380, can inhibit to 100%. This partial inhibition of BNPP cleavage is consistent with these compounds binding only partially in the competitive site/hydrophobic pocket. In contrast, PAT-352 and PAT-494 show distinct inhibition profiles against the artificial substrate, FS-3. Using FS-3 as the substrate, PAT-352 is an effective inhibitor, whereas PAT-494 shows no inhibition of FS-3 cleavage. Modeling of the FS-3/ATX structure showed that the FS-3 hydrocarbon chain occupies the hydrophobic pocket, and the phosphate portion of the molecule extends into the active site (Fells et al., 2013). As a result, compounds which bind in the active site/hydrophobic pocket would be expected to inhibit cleavage of FS-3 as shown for HA155 and PF-8380. Importantly, the modeling suggests that FS-3 does not bind in the hydrophobic channel, so compounds binding here would not be expected to inhibit FS-3 cleavage. This is consistent with the data obtained for PAT-347. With respect to the difference in FS-3 cleavage between PAT-352 and PAT-494, we hypothesize that occupying the PAT-494/352 (site 1) binding site alone is insufficient to inhibit FS-3 cleavage because these compounds do not bind in the active site and do not sufficiently occupy the hydrophobic pocket. However, for PAT-352, the backbone rearrangement occludes the entrance to the hydrophobic pocket, thus preventing binding of FS-3.

The X-ray structures obtained for the four compounds described provide the structural basis for their mechanism of inhibition and may provide guidance for the rational design of potent, selective ATX inhibitors for therapeutic benefit. In
addition, the ATX inhibitors and knowledge of the ATX-inhibitor complexes provides tools to study the in vitro and in vivo biology of ATX. This could include the binding of ATX to other proteins or membrane surfaces such as the binding of ATX to β3 integrins, which in turn mediates platelet interactions. Of particular interest is the role of the secondary AA binding site we identified on human ATX. This may represent a second LPC binding site that facilitates LPC migration into the catalytic site, or it may function as part of the LPA transfer mechanism to LPA receptors.

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