Autotaxin facilitates selective LPA receptor signaling

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SUMMARY

Autotaxin (ATX; ENPP2) produces the lipid mediator lysophosphatidic acid (LPA) that signals through disparate EDG (LPA1–3) and P2Y (LPA4–6) G protein-coupled receptors. ATX/LPA promotes several (patho)physiological processes, including in pulmonary fibrosis, thus serving as an attractive drug target. However, it remains unclear if clinical outcome depends on how different types of ATX inhibitors modulate the ATX/LPA signaling axis. Here, we show that the ATX "tunnel" is crucial for conferring key aspects of ATX/LPA signaling and dictates cellular responses independent of ATX catalytic activity, with a preference for activation of P2Y LPA receptors. The efficacy of the ATX/LPA signaling responses are abrogated more efficiently by tunnel-binding inhibitors, such as ziritaxestat (GLPG1690), compared with inhibitors that exclusively target the active site, as shown in primary lung fibroblasts and a murine model of radiation-induced pulmonary fibrosis. Our results uncover a receptor-selective signaling mechanism for ATX, implying clinical benefit for tunnel-targeting ATX inhibitors.

INTRODUCTION

Lysophosphatidic acid (LPA; mono-acyl-sn-glycero-3-phosphate) is a lipid mediator that signals through specific G protein-coupled receptors (GPCRs) to regulate multiple biological processes.1,2 Six LPA receptors (LPARs), belonging to two unrelated families, have been identified. LPA1–3 belong to the so-called EDG family, together with the receptors for sphingosine-1-phosphate (S1PR1–5), whereas LPA4–6 are P2Y (purinergic-type) GPCRs.3 Strikingly, structural studies have revealed different modes of ligand (LPA) entry for the prototypic receptor, LPA1 (EDG2) versus that for LPA6 (P2Y5). LPA1 accepts its LPA ligand from the extracellular space (water phase), whereas LPA6 is thought to receive LPA by lateral diffusion, following insertion into the outer lipid bilayer.4,5 All LPARs activate diverse effector pathways by coupling to distinct heterotrimeric G proteins. The LPAR expression pattern and G protein coupling repertoire in a given cell type largely determine the signaling output, which includes proliferation and survival via AKT and ERK pathways, and migration and invasion via Rho-family small GTPases that drive actin-based cytoskeletal changes.6,7 LPA signaling is restrained by the action of cell-associated lipid-phosphate phosphatases (LPP1–3)8 that dephosphorylate LPA to non-signaling monoacylglycerol.

LPA is produced by autotaxin (ATX or ENPP2), a secreted lyso-phospholipase D (lysoPLD) that hydrolyzes extracellular lysosphatidylcholine (LPC) and other lysophospholipids into bioactive LPA.9 ATX is secreted by diverse cell types and is present in body fluids; it is the only member of the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) family with lysoPLD activity.10 The ATX-LPAR signaling axis regulates numerous biological activities, including embryonic development and postnatal organ function,11 and has been implicated in life-threatening diseases, particularly pulmonary fibrosis and cancer.13,14 For this reason, ATX has attracted considerable interest as a drug target.

ATX is a compact, multi-domain glycoprotein consisting of a central bimetallic catalytic phosphodiesterase (PDE) domain flanked by two N-terminal somatomedin B-like domains and a C-terminal inactive nuclease-like domain.15,16 The PDE domain has a tripartite binding site that has been the focus of drug discovery and development.17 This tripartite site is composed of (1) a catalytic bimetallic site next to a hydrophilic shallow groove that accommodates the glycerol moiety of lipid substrates, (2) a hydrophobic pocket that binds acyl chains, and (3) a partially hydrophobic tunnel in a T-junction leading to the other side of the PDE domain.15,16 Importantly, the tunnel binds LPA16 as well as steroid molecules,18 and has been proposed to serve as an “exit channel” that might also modulate catalytic efficiency.16,19
**Table 1. Crystallographic details**

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aHigh-resolution shell in parentheses.

ATX interacts with cell-surface integrins and/or heparan sulfate proteoglycans and has been speculated to directly interact with LPARs. Although evidence for the latter notion is lacking. Through interaction with the cell surface, ATX may facilitate delivery of LPA to its cognate GPCRs in a highly localized manner. Recent studies have revealed ATX/LPA as a T cell repellent and suggested that ATX secreted by tumor cells functions as a LPA-producing “chaperone” that protects LPA from rapid degradation.

In recent decades, many small-molecule ATX inhibitors have been developed that show different ATX binding modes with respect to occupying the catalytic site and the hydrophobic pocket together (type I), or the pocket alone (type II), or the tunnel alone (type III). The first ATX inhibitor to enter clinical trials for idiopathic pulmonary fibrosis (IPF), the clinical candidate ziritaxestat (GLPG1690) designed by Galapagos (Mechelen, Belgium), is a hybrid molecule that occupies both the hydrophobic pocket and the tunnel and was defined as a type IV inhibitor. Subsequent ATX inhibitors to enter clinical trials for fibrotic diseases and tumor progression are either type III or type IV. It remains unclear whether the ATX binding mode of the different inhibitor types determines physiological outcome and, particularly, the function of ATX’s unique tunnel therein.

Here, we address these questions by showing that only tunnel-blocking ATX inhibitors are capable of inhibiting the full spectrum of LPA-mediated signaling responses. Furthermore, we demonstrate that an intact tunnel is essential for this effect. Strikingly, ATX/LPA-mediated signaling shows a strong preference for P2Y family GPCRs (LPAR), which accept the LPA ligand from the lipid bilayer. Finally, we validate these findings in lung fibroblasts and in a murine model of radiation-induced pulmonary fibrosis. Taken together, our results show that the ATX tunnel is essential for transport and delivery of LPA to LPARs, resulting in enhanced and selective signaling outputs. Thus, by virtue of the ATX tunnel, ATX-bound LPA functions as a “biased” receptor agonist. These findings bring a new perspective in our understanding of the ATX-LPA signaling axis and highlight the clinical benefit of targeting the ATX tunnel to block LPA signaling.

**RESULTS**

Different types of ATX inhibitors modulate select signaling events

We analyzed two highly potent compounds inhibiting LPC hydrolysis: a newly developed type I inhibitor, termed compound A (CpdA), and the type IV inhibitor ziritaxestat (GLPG1690). The crystal structures of CpdA and ziritaxestat bound to rat ATX confirmed their expected binding modes (see Table 1 for crystallographic data). CpdA is a classic competitive type I inhibitor, occupying the active site and hydrophobic pocket and forming several hydrogen bonding interactions, while ziritaxestat is a type IV inhibitor binding the tunnel, mostly by van der Waals interactions, as previously shown in complex with murine ATX (Figures 1A–1C and S1A–S1D). As the tunnel is a secondary binding site for LPA, which can modulate LPC hydrolysis, we confirm that ziritaxestat, but not CpdA, competes with LPA for tunnel occupancy (Figure S1E).

Having verified the structural binding mode for both compounds, we examined whether these distinct inhibitors behave differently in a cellular context. The effects of CpdA and ziritaxestat, in addition to PF8380 (a type I inhibitor) and Cpd17 (a type IV inhibitor), were therefore assessed. Type I compounds were more efficient in inhibiting ATX catalytic activity in vitro (apparent half maximal inhibitory concentration [IC<sub>50</sub> app] < 10 nM) than type IV compounds (IC<sub>50</sub> app > 25 nM) (Figure 1D). However, type I and type IV compounds were equally proficient in inhibiting ATX signaling in cell culture (apparent half maximal effective concentration [EC<sub>50</sub> app] ~ 1 μM), as measured using western blot analysis of AKT activation in human BJeH skin fibroblasts (Figure 1D). The lack of correlation between inhibition of catalytic efficiency in vitro and cellular outcome suggests that occupying the ATX tunnel could determine biological outcome.

To corroborate these findings, we wanted to examine the effect of ATX in an ATX-free extracellular environment. Therefore, we evaluated ATX expression (using quantitative polymerase chain reaction [qPCR]) and secretion (using western blotting) in several cell lines (Figures 1E and S1F). As NIH 3T3 fibroblasts were found to lack detectable ATX expression, as reported previously, these cells were used in subsequent experiments.
Figure 1. Different ATX inhibitors differentially modulate cellular signaling

(A and B) Schematic of (A) type I and (B) type IV modes of binding. Binding pose of CpdA and ziritaxestat at the ATX tripartite site from co-crystal structures (PDB: 7Z3K, 7Z3L) are shown on the right.

(C) Surface representation of the ATX tripartite site. The binding poses of CpdA (in green) and ziritaxestat (in orange) in each co-crystal structure have been superimposed.

(D) Inhibition of ATX activity in vitro and in BJeH cells; LysoPLD activity of ATX (20 nM), measured by choline release from LPC(18:1) (150 μM), and the inhibitory effect of four different ATX inhibitors, indicated as apparent IC\textsubscript{50} and K\textsubscript{i} values, measured at 0.05% w/v (7.5 μM) fatty acid-free (FAF) bovine serum albumin (BSA). The effect of type I (CpdA) and type IV (ziritaxestat) inhibition on ATX-mediated LPC(18:1) hydrolysis in BJeH human skin fibroblasts was examined using western blot analysis of p-AKT. The inhibitory effect of four different ATX inhibitors is indicated as apparent IC\textsubscript{50} and K\textsubscript{i} values, and EC\textsubscript{50} values, respectively.

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Cells were stimulated using recombinant ATX, with or without LPC substrate, alone or with ATX inhibitors CpdA or ziritaxestat. Phosphorylation of AKT and ERK was more strongly inhibited by ziritaxestat than CpdA (Figure 1F). We note that p-AKT or p-ERK was reduced in absolute levels (normalized to total actin) and in comparison with total AKT or ERK levels (in subsequent experiments only normalized values are reported). Moreover, ziritaxestat markedly abrogated RhoA activation, whereas the impact of CpdA was limited (Figure 1G). As a phenotypical readout of RhoA activation, ATX-induced trans-well cell migration was increased by LPC (18:1) and was more efficiently reduced by ziritaxestat than by CpdA (Figure 1H).

The feature that distinguishes ziritaxestat from CpdA is not its potency in inhibiting LPC hydrolysis, but its occupancy of the LPA-binding tunnel. This suggests that specific LPA signaling functions may be mediated through LPA binding to the ATX tunnel. We therefore examined if ATX can act as an LPA chaperone, independent of its catalytic activity.

**ATX is a dual-function protein that acts as an LPA chaperone in LPA signaling**

LPA is known to activate AKT, ERK, and RhoA through distinct G protein-effector pathways. Of note, in all subsequent experiments, we used FAF albumin (a known LPA carrier) at a concentration of 7.5 μM (0.05% w/v), much higher than the concentration of recombinant ATX (20 nM). Albumin-bound LPA efficiently activated ERK in NIH 3T3 cells, but only marginally activated AKT and RhoA (Figures 2A and 2B). Addition of recombinant ATX alone activated AKT, RhoA, and, to a lesser extent, ERK (Figures 2A and 2B). It therefore appears that both LPA in the presence of albumin and ATX alone (without added LPA) activate complementary pathways. Furthermore, ATX preincubated with LPA (ATX-bound LPA) significantly enhanced the activation of AKT and RhoA, but not ERK, compared with stimulation with LPA or ATX alone (Figures 2A, 2B, S2A, and S2B). In cell migration assays, NIH 3T3 cells failed to respond to albumin-bound LPA, but did respond to ATX-bound LPA (Figure 2C). Similar results were obtained using ATX-deficient MDA-MB-231 breast carcinoma cells (Figure S2C). However, in ATX-secreting RAT-1 and BJeH fibroblasts, albumin-bound LPA was sufficient to activate both AKT and ERK; this activation was not enhanced by adding ATX (Figures 2D and S2D). These results suggest that ATX in the extracellular milieu mediates activation of AKT and RhoA through LPAR signaling.

Next, we examined if the effect of exogenously added ATX could be recapitulated by enforced ATX expression in NIH 3T3 cells. Transient ATX expression enabled LPA-induced activation of AKT but reduced ERK activation in this setting (Figure 2E); this recapitulated the effect of LPA observed in BJeH and RAT-1 cells, which are endogenously expressing ATX.

This suggests that ATX is needed for specific aspects of LPA signaling. As binding of ziritaxestat to the tunnel was important for abrogating the activation of AKT, ERK, and RhoA in the context of ATX-mediated LPC hydrolysis (Figures 1F–1H), we asked if ziritaxestat or CpdA affect the action of ATX-bound LPA. Ziritaxestat, but not CpdA, inhibited AKT and ERK activation by ATX-bound LPA (Figures 2F and S2E). Consistently, ziritaxestat showed a stronger effect than CpdA on LPAR-driven RhoA activation (Figure 2G). Both compounds affected NIH 3T3 cell migration; however, ziritaxestat exerted a more pronounced effect (Figure 2H), which was not significantly different from that of CpdA.

Taken together, these results suggest that type IV inhibitors occupying the ATX tunnel do more than inhibit LPA production. According to this scenario, ATX is a dual-function protein that acts as an LPA-producing chaperone with functional specificity.

**LPA delivery by ATX requires a structurally intact tunnel**

To decouple the catalytic activity of ATX from its chaperone signaling function, we performed a series of mutations (Figure 3A): the catalytic nucleophile Thr210 was mutated to alanine to abrogate ATX lysoPLD activity; residue Gly257 was mutated into a lysine to bring a positive charge and a long side chain hampering entry of LPA molecules from the back of the tunnel; and residue Trp255 located in the middle of the LPA-binding tunnel was mutated to alanine, removing such potential aromatic-aliphatic interactions which have been observed with LPA,18 oxysterols18 and inhibitors.27 Although both ATX(T210A) and ATX(W255A) mutants rendered ATX catalytically inactive in vitro, ATX(G257K) retained ~30% of the catalytic activity (Figure 3B).

We then asked if the tunnel mutants would affect LPA binding. To assess this we performed a series of molecular dynamics (MD) simulations on wild-type ATX and the three mutants, as described previously.19 The observed LPA binding events in the tunnel were reduced in all three mutants, compared with wild-type (Figure 3C). Whereas LPA binding in the tunnel was observed in 50% of the simulations on wild-type ATX, ATX(T210A) showed binding in 20% of the simulations and ATX(G257K) in 10% of the cases. No binding events were observed for ATX(W255A). Thus, we have created (1) a catalytically inactive mutant that has a compromised but functioning...
Figure 2. Role of ATX in LPA-mediated signaling and effect of distinct ATX inhibitors

(A) ATX as an LPA carrier driving activation of AKT and ERK in NIH 3T3 cells. Cells were serum starved for 16 h and stimulated for 5 min with albumin-bound LPC (1 μM) or LPA (1 μM), with ATX alone (20 nM) or with ATX preincubated for 30 min with LPC or LPA. Albumin-bound LPA was used as a control for NIH 3T3 cells after a 2.5 min stimulation and was used for normalization. Left panel: representative western blot; right panels: quantitation of AKT and ERK activation.

(B) RhoA activation in NIH 3T3 cells in response to the indicated stimulants. The response amplitude was quantitated (see Figure S2 for complete time course). Data depict median ± IQR of 20 fields containing at least 10 cells.

(C) Trans-well migration of NIH 3T3 cells in a Boyden chamber in serum-free media, using a gradient of albumin- or ATX-bound LPA(18:1) as stimulant, where the concentrations of the carriers remained constant. Left panel: quantitation of LPA-dependent cell migration depending on the carrier; right panel: representative filter containing fixed cells. Albumin alone does not suffice to enable LPA-mediated cell migration.

(D) ATX as an LPA carrier driving activation of AKT and ERK in NIH 3T3 cells. Cells were serum starved for 16 h and stimulated for 5 min with albumin-bound LPC (1 μM) or LPA (1 μM), with ATX alone (20 nM) or with ATX preincubated for 30 min with LPC or LPA. Note the contrasting pattern of p-AKT to that of (A). Left panel: representative western blot; right panels: quantitation of AKT and ERK activation.

(E) Need of endogenous ATX production for AKT activation in NIH 3T3 cells. Cells were serum starved and transfected with 1 μg ATX cDNA for 24 h. Left panel: representative western blot; right panels: quantitation of AKT and ERK activation.

(F) The ability of CpdA and ziritaxestat to compete with LPA for binding to ATX was tested in NIH 3T3 cells. Cells were treated with ATX-bound LPA for 5 min in the presence or absence of CpdA (5 μM) or ziritaxestat (5 μM) and compared with albumin-bound LPA (control). Left panel: representative western blot; right panels: quantitation of AKT and ERK activation.

(G) Ziritaxestat is more efficient than CpdA in inhibiting ATX-LPA-driven RhoA activation in NIH 3T3 cells in response to ATX-bound LPA for 10 min in the presence or absence of CpdA (5 μM) or ziritaxestat (5 μM). Data depict median ± IQR of 20 fields containing at least 10 cells. Data in (A), (C),–(F), and (H) represent the mean value of triplicate biological measures ± SEM (error bars). For all panels, *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not significant (one-way ANOVA, Tukey’s post hoc test; when not specified, tested versus negative control using Dunnett’s post hoc test).
A Wildtype ATX  Catalytic and tunnel mutants  Surface of tripartite site
Trp256  Gly257  Thr210  W255A  G257K  T210A

B LysoPLD activity
Wildtype ATX  T210A  G257K  W255A  Background
Choline (μM)
0  20  40  60  80  100
Time (min)

C RMSD (nm)
Time (ns)

D p-AKT  p-ERK  Actin

E RhoA
YFP/CFP Ratio

F Transfection
ATX  G257K  W255A
LPA  +  +  +

G Cell motility

(legend on next page)
tunnel (T210A), (2) a mutant that retains catalytic activity but has a compromised tunnel (G257K), and (3) a mutant that lacks both catalytic activity and a functional tunnel (W255A). We then assessed these mutants in cell-based assays to better understand the importance of an intact tunnel for downstream LPA signaling.

Preincubating all ATX variants (wild-type, T210A, G257K, W255A) with LPA resulted in significantly increased AKT activation by wild-type ATX, ATX(T210A), and ATX(G257K), but not by ATX(W255A) (Figures 3D, S2A, and S3A–S3F), compared with LPA alone. ERK activation was not significantly different in the presence of wild-type ATX or any of the mutants (Figures 3D and S3A–S3F). Catalytically inactive ATX(T210A) activated RhoA, albeit to a lesser extent than wild-type ATX; by contrast, the ATX(G257K) and ATX(W255A) tunnel mutants failed to activate RhoA (Figures 3E and S3G). Additionally, in NIH 3T3 cell migration assays, both wild-type ATX and ATX(T210A), but not ATX(W255A), induced chemotactic activity when preincubated with LPA (Figure 3F). ATX(G257K) showed an intermediate phenotype and required higher LPA concentrations to cause cell response. These results indicate that the ATX tunnel is key for specific signaling functions dependent on ATX and are not coupled to catalytic activity.

We then asked if endogenously secreted ATX could recapitulate the signaling outcome triggered by recombinant protein. Transient transfection of the respective ATX variants in NIH 3T3 cells led to ATX secretion and enabled LPA-induced AKT activation (Figure 3G). Although ATX(T210A) has a similar effect to that of wild-type ATX, the two tunnel mutants did not significantly increase the activation of AKT with respect to LPA alone. However, none of the overexpressed ATX variants resulted in a significant increase of ERK activation.

Functional selectivity of ATX: Preference for P2Y-type LPAs over EDG-type LPA

Given that LPA1-3 and LPA4-6 are evolutionarily distinct receptors and use different ligand entry modes (Figure 4A), we asked whether ATX/LPA shows LPAR selectivity. As HeLa-Flp-In cells exhibited very low (or undetectable) expression of ATX and LPA1-6 (Figure 1D), we used these cells to reconstitute inducible expression of EDG LPA1 and P2Y LPA6 (C-terminally HA tagged) and to analyze ATX/LPA signaling. We confirmed that both LPA1 and LPA6 were expressed at similar levels, as shown by qPCR and western blot analyses (Figures S4A and S4B), and were localized to the plasma membrane, as shown by confocal microscopy (Figure S4C).

We used these cell lines to examine receptor activation in response to ATX, LPA, and ATX preincubated with LPA. As a readout, we used agonist-receptor internalization and quantified cytoplasmic LPAR-containing vesicles by confocal imaging. LPA1 was internalized in response to albumin-bound LPA and ATX-bound LPA, but to a lesser extent in response to ATX alone (Figure 4B). By contrast, LPA6 did not exhibit detectable internalization upon LPA stimulation, but responded more strongly to ATX and to ATX preincubated with LPA (Figure 4C). This suggests that the non-catalytic effects of ATX are preferentially mediated by the P2Y-type LPARs than by EDG-type LPARs.

Consistent with this, LPA1-expressing HeLa cells responded to albumin-bound LPA, as shown by activation of AKT, ERK, and RhoA, irrespective of ATX presence (Figures 4D, 4E, S4D, and S4E). In marked contrast, LPA6-expressing cells showed much stronger AKT/ERK/RhoA activation responses to ATX-bound LPA than to albumin-bound LPA (but weaker than to ATX alone) (Figures 4F, 4G, S4D, and S4E).

Finally, we examined if CpdA and ziritaxestat differentially affected LPA1- versus LPA6-mediated signaling in induced HeLa cells. We focused on the dual function of ATX, using ATX preincubated with LPA. In LPA1-expressing cells, both CpdA and ziritaxestat reduced the internalization of LPA1 and activation of RhoA (Figures 4H and 4I). In LPA6-expressing cells, both compounds reduced LPA6 internalization (Figure 4J), but ziritaxestat was a more efficient inhibitor of RhoA activation (Figure 4K).
Collectively, these findings suggest that ATX-bound LPA signaling shows preference for P2Y LPARs. Given the specific effect of ziritaxestat and its therapeutic potential for lung fibrosis, we aimed to corroborate the above mechanism in primary human lung fibroblasts.

**ATX-mediated LPA delivery in human lung fibroblasts**

Normal human lung fibroblasts (NHLFs) express and secrete ATX and express high levels of EDG receptors LPA1, and LPA6, and P2Y receptor LPA6 (Figure 1D).

Treatment of NHLFs with albumin-bound LPA resulted in weak activation of AKT, but activated ERK (Figures 5A and SSA). Even though non-activated NHLFs secrete ATX (Figure 1E), the response of activated NHLFs to LPA resembles more the ATX-free NIH 3T3 cells (Figure SSA). ATX alone, ATX plus LPC, and ATX-bound LPA activated both AKT and ERK. This is in agreement with the results from NIH 3T3 cells (Figure 2A). Stimulation with catalytically inactive ATX(T210A) resulted in AKT activation, whereas stimulation with the mutant ATX(W255A) failed to do so (Figure SSB). CpdA did not significantly inhibit AKT or ERK activation (Figure 5B), and only affected the RhoA response to ATX-bound LPA to a low extent (Figures 5C and SCC). By contrast, tunnel-binding ziritaxestat abrogated AKT and RhoA activation, confirming that our results extend to early-passage NHLFs.

As all cell-based assays confirmed that ATX is a dual-function protein, and that ziritaxestat (but not CpdA) inhibits its activity as an LPA chaperone with functional specificity (Figure 3D), we then examined the relative efficacy of ziritaxestat and CpdA in vivo, in a murine model of pulmonary fibrosis.

**Tunnel-blocking ziritaxestat, but not CpdA, reverses pulmonary fibrosis in mice**

Ziritaxestat and CpdA were evaluated in a model of radiation-induced pulmonary fibrosis, a serious adverse effect of radiotherapy for patients with lung cancer with few treatment options, and relevant for progressive lung fibrosis in a therapeutic setting (Figure 6A). The beneficial outcome of ziritaxestat on radiotherapy has been assessed in previous work; in this mouse model both compounds were evaluated in two separate experiments, where the doses were selected on the basis of the compounds’ potency in rat plasma LPA assays (Figure 6B). Drug exposure was measured at steady state in rat plasma LPA assays, which confirmed the similar target coverage and respective IC50 values of the compounds (541 nM for ziritaxestat and 13.6 nM for CpdA) (Figure 6B; Table S1). When assayed above their IC50 values, CpdA was more efficient than ziritaxestat in inhibiting LPA production (Figure 6C).

As fibrotic areas are characterized by increased secretion of extracellular matrix proteins, including type I and III collagen, we quantitated the effect of CpdA and ziritaxestat on reducing pulmonary collagen I levels. Irradiation induced a significant increase in collagen I levels after 19 weeks compared with the non-treated control, which was reduced in both experiments by treatment with nintedanib, a clinically approved drug for the treatment of IPF (Figures 6D and 6E).3,3 While CpdA treatment did not decrease collagen I levels significantly (p = 0.59), ziritaxestat treatment showed some attenuation of collagen I secretion (p = 0.048) (Figures 6D and 6E). Pharmacokinetic analysis of lung exposure after single oral administration of both compounds (at 10 mg/kg) showed that the values for the area under the concentration-time curve from 0 to 6 h (AUC0-6h) were 5-fold higher for CpdA than for ziritaxestat (6,123 versus 1,427 ng · h/g) (Figure 6F). As such, the lack of CpdA therapeutic activity was not due to lower pulmonary exposure.

In summary, targeting the ATX tunnel and the related non-catalytic ATX activities is important both in vitro and in vivo, highlighting the therapeutic potential of ATX inhibitors that block the tunnel and its chaperone function.

**Figure 4. ATX shows a preference for P2Y-type LPA over EDG-type LPA**

(A) Surface representation of the differing entry modes of LPA(18:1) into the binding pockets of LPA1 (left) and LPA6 (right). UCSF ChimeraX 1.2.5 was used to generate surfaces. Membranes with bound LPARs were created and rendered using Blender 2.93.5. PDB codes. The structures were retrieved from the AlphaFold database (AF-Q92633-F1 [LPA1] and AF-P43657-F1 [LPA6]29,30).

(B and C) Representative images and quantitation of the internalization of (B) LPA1-HA and (C) LPA6-HA in HeLa-Flp-In cells upon stimulation with albumin-bound LPA (1 μM), ATX (20 nM), or ATX-bound LPA for 15 min. Left panels: representative confocal images used for vesicle quantitation; right panel: calculation of the number of internalized vesicles.

(D) Activation of AKT and ERK in LPA6-HA-expressing HeLa cells. Stimulation of LPA6-HA in HeLa-Flp-In cells that were starved overnight with 0.5% serum-containing medium, where receptor expression was also induced. Top panel: representative western blot; bottom panels: quantitation of AKT and ERK activation.

(E) RhoA activation in response to albumin-bound LPA (1 μM), ATX (20 nM), and ATX-bound LPA in HeLa-Flp-In cells (YFP/CFP fluorescence ratio) mediated by LPA6-HA. The response amplitude was quantitated (see Figure 4 for full time course).

(F) Activation of AKT and ERK in LPA6-HA-expressing HeLa cells. Stimulation of LPA6-HA in HeLa-Flp-In cells that were starved overnight with 0.5% serum-containing medium, where receptor expression was also induced. Top panel: representative western blot; bottom panels: quantitation of AKT and ERK activation.

(G) RhoA activation in response to albumin-bound LPA (1 μM), ATX (20 nM), and ATX-bound LPA in HeLa-Flp-In cells (YFP/CFP fluorescence ratio) mediated by LPA6-HA. The response amplitude was quantitated (see Figure 4 for full time course).

(H) Induction of the internalization of LPA1-HA in HeLa-Flp-In cells by ATX-bound LPA (20 nM, 1 μM) with or without CpdA (5 μM) or ziritaxestat (5 μM). Intracellular vesicle count upon a 15 min stimulation in the presence of serum-free Dulbecco’s modified Eagle’s medium (DMEM) containing 0.05% FAF-BSA.

(I) RhoA activation in LPA1-HA-expressing HeLa-Flp-In cells (shown as a YFP/CFP fluorescence ratio) upon stimulation with ATX-bound LPA for 10 min in the presence or absence of CpdA (5 μM) or ziritaxestat (5 μM).

(J) Induction of the internalization of LPA6-HA in HeLa-Flp-In cells by ATX-bound LPA (20 nM, 1 μM) with or without CpdA (5 μM) or ziritaxestat (5 μM). Intracellular vesicle count upon a 15 min stimulation in the presence of serum-free DMEM containing 0.05% FAF-BSA. Note that CpdA and ziritaxestat abolish vesicle internalization in LPA6-HA-expressing cells.

(K) RhoA activation in LPA6-HA-expressing HeLa-Flp-In cells (shown as a YFP/CFP fluorescence ratio) upon stimulation with ATX-bound LPA for 10 min in the presence or absence of CpdA (5 μM) or ziritaxestat (5 μM).

Data in (B)-(K) represent the mean value of triplicate biological measurements ± SEM (error bars), and in (B), (C), (H), and (J) at least 20 fields were analyzed. Data in (E), (G), (I), and (K) depict median ± IQR of 20 fields containing at least 10 cells. For (B)-(K), *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not significant (one-way ANOVA, Tukey’s post hoc test; when not specified, tested versus negative control using Dunnett’s post hoc test).
DISCUSSION

Following its implication in tumor progression and pulmonary fibrosis, ATX has attracted considerable interest as a drug target from pharmaceutical companies and academic researchers. Structural studies demonstrated that ATX inhibitors exhibit different binding modes. ATX inhibitors entering clinical trials in patients with pulmonary fibrosis or cancer include ziritaxestat, structural studies demonstrated that ATX inhibitors exhibit different binding modes. ATX inhibitors entering clinical trials in patients with pulmonary fibrosis or cancer include ziritaxestat,

Figure 5. ATX-mediated LPA delivery and signaling in human lung fibroblasts

(A) Stimulation of NHLFs with LPA or LPC in the presence or absence of ATX for 10 min. Top panel: representative western blot; bottom panels: quantitation of AKT and ERK activation.

(B) Stimulation of NHLFs with LPA, ATX, or ATX-LPA with or without CpdA or ziritaxestat for 10 min. Top panel: representative western blot; bottom panels: quantitation of AKT and ERK activation.

(C) RhoA activation in response to albumin-bound LPA, ATX, or ATX-bound LPA in the presence or absence of CpdA or ziritaxestat. The response amplitude was quantitated (see Figure S5 for full time course). Data depict median ± IQR of 20 fields containing at least 10 cells.

(D) ATX serving as an LPA-producing chaperone, in a schematic representation showing secreted ATX with its tripartite site, LPA binding in the ATX tunnel, and the binding modes of ATX inhibitors CpdA and ziritaxestat. LPA1 accepts LPA from the water phase, whereas LPA6 accepts LPA from the lipid bilayer (Figure 4A), with ATX serving as an LPA-producing chaperone.

Data in (A) and (B) represent the mean value of triplicate biological measures ± SEM (error bars). For (A)–(C), *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not significant (one-way ANOVA, Tukey’s post hoc test; when not specified, tested versus negative control using Dunnett’s post hoc test).
which progressed to phase 3. BBT-877 (Bridge Biotherapeutics, Pangyo-ro, South Korea), IOA-289 (iOnctura, Geneva, Switzerland), and cudetaxestat (Blade Therapeutics, South San Francisco, CA) are now in phase 1 and 2 trials and are also type IV (or perhaps, for some, type III) inhibitors, displaying the common characteristic of occupying the ATX tunnel. None of the numerous potent type I orthosteric ATX inhibitors occupy the tunnel, and none have entered clinical trials, to the best of our knowledge. These data raise the intriguing questions of whether and how the specific drug binding mode determines the physiological outcome of ATX/LPA signaling, both in vitro and in vivo.

Here we have addressed this question by examining how type I and type IV ATX inhibitors affect specific LPAR-mediated

Figure 6. Ziritaxestat, but not CpdA, reverses pulmonary fibrosis in vivo

(A) Radiation-induced fibrosis model. Irradiated lung was treated with CpdA (10 mg/kg b.i.d.), ziritaxestat (30 mg/kg b.i.d.), or nintedanib, a clinically approved drug for the treatment of IPF (positive control; 60 mg/kg q.d.).

(B) Percentage of reduction in 18:2 LPA formation from ex vivo rat plasma samples upon incubation with CpdA or ziritaxestat, as determined by liquid chromatography-mass spectrometry. The percentage of inhibition was calculated by normalization of data to LPA levels after a 2 h incubation at 37°C in the absence of the compounds. The resulting IC_{50} values (±SEM) are shown.

(C) Mean (±SEM) steady-state plasma concentrations of CpdA and ziritaxestat after a p.o. administration at 10 mg/kg b.i.d. and 30 mg/kg b.i.d., respectively, shown on logarithmic scale.

(D and E) Analysis of collagen I levels in the murine model of radiation-induced fibrosis. (D) Representative images of collagen I staining. (E) Surface area quantitation of collagen I levels of CpdA- or ziritaxestat-treated mice after 19 weeks. *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not significant (one-way ANOVA, Dunnett’s post hoc test).

(F) Lung tissue exposure of CpdA and ziritaxestat after a single p.o. administration at 10 mg/kg.
signaling events. We find that type IV compounds are more efficient in inhibiting AKT activation, RhoA activation, cell migration, and receptor internalization. Strikingly, our data suggest that ATX is not only needed for LPA production, but that it can also act as an LPA chaperone. It is this specific chaperone activity that can be targeted by type III or type IV compounds, which occupy the ATX tunnel. We corroborate these findings in fibroblast cell lines, LPA1- and LPA6-expressing HeLa cells, NHLFs, and in a murine model of radiation-induced pulmonary fibrosis.

Our findings support the view that ATX acts as a dual-function protein that can activate signaling pathways by virtue of its unique tunnel, independent of its catalytic activity. The LPA chaperone role has unique characteristics, but also analogies to other lipid chaperones. Albumin is the primary and most abundant LPA chaperone, showing high LPA binding affinity. In our experiments, the albumin concentration was ~8 µM. The chaperone function of ATX was prominent as low as 20 nM, in the presence of albumin as a “competitor” for LPA recruitment. Analogies with the S1P lysolipid chaperone are thus apparent. Serum albumin binds S1P, but with lower affinity than high-density lipoprotein (HDL)-associated apolipoprotein M (ApoM), which serves as the major circulatory S1P chaperone. It has been shown that the ApoM/S1P complex evokes S1PR1-mediated biological responses in a selective manner, distinct from those evoked by albumin-bound S1P. ApoM/S1P thus serves as a “biased agonist” for S1PR1. Unlike ApoM-bound S1P, albumin-bound S1P is short lived in plasma and activates S1PR1 in a manner distinct from that of ApoM-S1P. The present results for LPA and ATX point to a similar mechanism, with the distinct peculiarity of the dual enzymatic and chaperone role of ATX.

The additional role of ATX mediating LPA delivery involves specific LPA binding to the ATX tunnel, which is in agreement with a previous hypothesis and with biochemical evidence and MD simulations indicating that the tunnel acts as a secondary binding site. The tunnel mutants ATX(G257K) and ATX(W255A), both display attenuated binding of LPA in MD simulations, consistent with our experimental findings. The partially accessible site with t1/2 that can be targeted by type III or type IV compounds, which act as an LPA chaperone. It is this specific chaperone activity that can be targeted by type III or type IV compounds, which occupy the ATX tunnel. We corroborate these findings in fibroblast cell lines, LPA1- and LPA6-expressing HeLa cells, NHLFs, and in a murine model of radiation-induced pulmonary fibrosis.

Our present data suggest that the physiological effects of ziraxestat, which showed promise in phase IIb trials, can be attributed to its binding mode occupying the ATX tunnel and thereby inhibiting specific signaling events including RhoA-mediated cytoskeletal reorganization.

At first sight, there is a discrepancy between the in vitro and in vivo effects shown for type I and type IV inhibitors, respectively. Although LPA1-deficient mice are protected from fibrosis in models of lung and dermal fibrosis, ATX expression is increased in the fibrotic skin of patients with systemic sclerosis and in a bleomycin mouse model of systemic sclerosis. Increased ATX and LPA levels have also been found in lung tissue and bronchoalveolar lavage fluid, respectively, of patients with IPF. Ziraxestat has been trialed as a treatment for IPF and systemic sclerosis, however, phase 3 trials in patients with IPF were discontinued because the benefit-risk profile of the drug no longer supported continuation. Our present data suggest that the physiological effects of ziraxestat, which showed promise in phase IIb trials, can be attributed to its binding mode occupying the ATX tunnel and thereby inhibiting specific signaling events including RhoA-mediated cytoskeletal reorganization.
of how lipid mediators, such as LPA, regulate biological functions in a receptor-selective manner and thus suggest new therapeutic opportunities. In a more general context, this study highlights how structural and mechanistic insights may translate into the (pre)clinical efficacy of inhibitors blocking the ATX tunnel. Several inhibitors that occupy the ATX tunnel are currently in clinical trials; this work provides a rationale for their mode of action, targeting both the enzymatic and the chaperone function of ATX. Future studies should also address to what extent P2Y LPAR signaling pathways contribute to lung fibrosis, either directly or indirectly.

Limitations of the study
Although ziritaxestat significantly reduced collagen I levels in irradiated mouse lungs and ameliorated murine disease outcome, the present experimental setting does not allow further comparison between ziritaxestat and CpdA (an inhibitor that does not target the ATX tunnel and, thus, the LPA chaperone function), as the mouse studies were performed in two different experiments. We cannot exclude that this experimental design results in considerable variation within the different groups, thereby hampering a direct comparison with the same reference groups. Additionally, the $K_i$, $IC_{50}$, and $EC_{50}$ values derived from our kinetic analyses should be understood as apparent, as bulk concentrations of the different inhibitors were used instead of the relative concentrations of their active physical forms.

SIGNIFICANCE
ATX produces the lipid mediator LPA, which regulates multiple biological functions via distinct GPCRs, termed LPA₁₋₆, belonging to different receptor families (EDG versus non-EDG or P2Y-type). The ATX-LPAR signaling axis has been implicated in life-threatening diseases, notably pulmonary fibrosis, liver disease, and cancer, and hence has emerged as an important therapeutic target. The catalytic domain of ATX is characterized by a tripartite binding site, showing a unique open tunnel adjacent to the active site and the hydrophobic substrate-binding pocket. Here we show that LPA binding in the tunnel defines ATX as an LPA-producing binding protein (or “chaperone”) that facilitates aspects of LPA receptor signaling, in cell-based assays. This chaperone function of ATX is abrogated by both tunnel-binding inhibitors and tunnel mutants. Interestingly, ATX-bound LPA shows a preference for P2Y-type LPA₆, which accepts LPA from the lipid bilayer rather than the aqueous phase as does EDG-type LPA₁. In addition, we show that occupancy of the ATX tunnel is essential for clinical candidate ziritaxestat (GLPG1690) to inhibit pulmonary fibrosis in a mouse model. These results provide new insights into ATX as a dual-function protein and, furthermore, imply therapeutic benefits for tunnel-targeting (type IV) ATX inhibitors, some of which are undergoing clinical trials.

STAR★METHODS
Detailed methods are provided in the online version of this paper and include the following:

- Lead contact
- Materials availability
- Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Cell culture
  - Animal model
- METHOD DETAILS
  - Crystallographic methods
  - Biochemical methods
  - Cell-based assays
  - MD simulations
  - Animal studies
  - Organic chemistry methods
  - LC chromatogram and Mass spectrum of ziritaxestat
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.chembiol.2022.12.006.

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AUTHOR CONTRIBUTIONS
Conceptualization, F.S.-P. and A.P.; Formal Analysis, F.S.-P., R.B., and M.-T.M.; Investigation, F.S.-P., R.B., and M.-T.M.; Instruct-NL, Instruct-ERIC center. We thank Elsa Matas-Rico and Willem-Jan Keune for help establishing cell-based experiments and Kees Jaal for discussions, ideas, and reagents. This study was partially funded by Galapagos NV (Mechelen, Belgium). Editorial support was provided by Iain Haslam (Aspire Scientific, Bollington, UK), funded by Galapagos NV. This research was supported by an institutional grant of the Dutch Cancer Society and of the Dutch Ministry of Health, Welfare and Sport.

DECLARATION OF INTERESTS
W.H.M. has consulted for Merck KGaA (Darmstadt, Germany) and iOctura SA (Geneva, Switzerland) on development of ATX inhibitors for use in cancer treatment. B.H. is an employee of Galapagos, a co-inventor of a patent related to ziritaxestat, and the owner of Galapagos subscription rights. F.M., C.J., and L.W. are employees of Galapagos. P.F. is a former employee and warrant holder at Galapagos. M.-T.M. is a co-founder of Cloudbpharm, an early-stage drug discovery company. A.P. has an adjunct professor appointment at the University of Utrecht, F.S.-P. and R.B. declare no competing interests.

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REFERENCES


**STAR METHODS**

**KEY RESOURCES TABLE**

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(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed and will be fulfilled by the lead contact, Anastassis Perrakis (a.perrakis@nki.nl).

Materials availability
Plasmids and cell lines generated in this study are available by request to the lead contact and are subject to signing a materials transfer agreement (MTA).

Data and code availability
- Crystallographic data have been deposited at the Protein Data Bank and are publicly available as of the date of publication. The co-crystal structures of ATX bound to CpdA or zirxstat are under codes 7Z3K and 7Z3L, respectively.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture
NIH-3T3 (CRL-1658, mouse; sex unknown); HeLa-Flp-In, HeLa-Flp-In-LPAR1-HA and HeLa-Flp-In-LPAR6-HA (derived from,54 human; female); BJeH (CRL-4001, human; male); RAT-1 (CVLC-492, rat; sex unknown); MDA-MB-231 (HTB-26, human; female); NHLF (CC-2512B, human; male); HEK-293T Flp-In (R75007, human; female). NHLF cells were cultured in FGM™-2 fibroblast growth medium supplemented with 2% fetal bovine serum (FBS), 0.1% insulin, 0.1% hFGF-B, and 0.1% GA-100. All other cell lines were cultured in DMEM supplemented with 10% (v/v) FBS, penicillin (50 U/mL), and streptomycin (50 mg/mL) at 37°C in 5% CO₂.

Animal model
Procedure and drug-naïve seven-week-old female B6 albino (B6N-Tyr<sup>C</sup>-Brd/BrdCr) mice (16–18 g) were obtained from Charles River (France) and delivered at the Plateforme de Radiothérapie Expérimentale in Institut Curie (Orsay, France).

METHOD DETAILS

Crystallographic methods
Purification of recombinant rat ATX
Rat ATX for structural studies was overexpressed and purified as described previously.59 Briefly, HEK-293T Flp-In cells were grown in DMEM medium containing 10% FBS, L-glutamine (2 mM), penicillin (50 U/mL), and streptomycin (50 mg/mL) at 37°C in 5% CO₂. Cells were then expanded and grown in the presence of 1% FBS for four days. Next, the culture medium was collected and centrifuged at 4,000 rpm for 15 min. The supernatant was then filtered through a 0.65-μm bottle-top filter, and applied onto a 10-mL Ni²⁺-loaded POROS-20 MC column at a flow rate of 1–2 mL/min. The column was washed with a buffer containing 20 mM Hepes and 150 mM NaCl, pH 7.4, and the protein was eluted with 2–3 column volumes of a linear gradient of buffer containing 1 M imidazole. The resulting fractions were applied onto a Superose 6 10/30 size-exclusion column, which were then pooled and concentrated.

Crystallization, X-ray data collection, structure solution and refinement
For the crystallization studies, ATX was incubated with each screened compound at a 1:10 (protein:compound) ratio for at least 30 min. Crystals were grown for a least 7 days in a 24-well optimization screen (18–20% PEG 3350, 0.1–0.4 M NaSCN, and...
0.1–0.4 M NH₄I). In all cases, the best diffracting crystals were obtained at room temperature (RT) by mixing 1 µL of the protein:compound solution and 1 µL of reservoir solution. All crystals were vitrified in cryoprotectant, which consisted of reservoir solution with the addition of 20% (v/v) glycerol. Other solvent:protein ratios tested per condition were 1:2 and 2:1.

The X-ray diffraction data for the ATX–inhibitor complexes were collected at SLS on beamline PXIII at 100 K, and were recorded on a PILATUS 2M-F detector to high resolution. All data were processed and integrated with XDS. CpdA and ziritaxestat were processed on site, using the SLS automated processing pipeline, and scaled with AIMLESS. The structures were determined by molecular replacement using MolReps and with the structure of ATX (PDB 2XR9) as the search model. Model building and subsequent refinement were performed iteratively with COOT, REFMAC5, and PDB_REDO. Structure validation was carried out by MolProbity. The structure models and experimental diffraction data of CpdA and ziritaxestat were deposited at the Protein Data Bank under the codes 7Z3K and 7Z3L, respectively (Table S2).

Biochemical methods

Biochemical analyses and modeling of kinetic data
The biochemical studies of ATX lysoPLD activity were performed with human ATX. Activity was measured by a coupled reaction with 1 U ml⁻¹ choline oxidase and 2 U ml⁻¹ horseradish peroxidase (HRP) and 2 mM homovanillic acid (HVA) (all from Sigma-Aldrich). For the assays, 150 µM 18:1 LPC (Avanti polar Lipids Inc.) was incubated with 20 nM ATX (unless otherwise stated), reaching a final volume of 100 µL buffer, which contained 50 mM Tris, 50 mM CaCl₂, 0.01% Triton X-100, 0.05% (w/v) (7.5 µM) fatty acid-free (FAF) bovine serum albumin (BSA), pH 7.4. Steady-state choline release was measured at 37°C by HVA fluorescence at λₐε/λₐε = 320/460 nm in Corning® 96- or 384-well OptiPlate (Sigma-Aldrich) and with a PHARstar plate reader (BMG Labtech). To determine the IC₅₀ for the different inhibitors on ATX activity, the velocity of the reaction was monitored for each compound as a function of time and the linear phase of the kinetics was taken from 60 min after the addition of ATX to the reaction buffer. The resulting fluorescence intensity signal over time was used to model all inhibitor concentrations simultaneously using the following formula, where Vmax and vmin were fitted for the minimum and maximum relative velocity, and ci corresponds to the inhibitor concentration for each assay:

\[ V = \frac{V_{\text{max}} - V_{\text{min}}}{(1 + c_i/IC_{50})} + V_{\text{min}} \]  

(Equation 1)

Competition with LPA allostery
The activation assays using LPA were performed in a similar way as those done for the inhibitors. In this case, LPA was dissolved in Ethanol: H₂O (1:1), 0.01% TX-100 and was added to the reaction buffer. The presence of ethanol was taken into account and controls in the absence of ATX and/or LPC were employed to correct the kinetic data. ATX was incubated for 30 min with different concentrations of inhibitors and subsequently added to the reaction buffer containing 150 µM 18:1 LPC and different starting concentrations of 18:1 LPA. The slopes were calculated from at least 60 min after the addition of ATX. The percentage of LPA-driven activation was normalized to ATX in the absence of LPA and inhibitors, which represented 100% activity. Lastly, the activation constant or AC₅₀ was obtained from the following equation:

\[ V = \frac{V_{\text{max}} - V_{\text{min}}}{(1 + c_i/AC_{50})} + V_{\text{min}} \]  

(Equation 2)

Cell-based assays

Purification of recombinant human ATX
Human ATX used in cell-based studies was overexpressed and purified as described previously with a procedure identical to the one briefly described for rat ATX in this manuscript under “crystallographic methods”.

AKT and ERK phosphorylation
A total of 300,000 NIH-3T3, 100,000 BJH, 100,000 RAT-1, 100,000 MDA-MB-231, 100,000 NHLF or 50,000 HeLa-Flp-In cells were seeded in 12-well tissue culture plates and allowed to grow for 24 h in DMEM (Gibco, Life Technologies) containing 10% fetal calf serum (FCS) (Thermo Scientific, USA) and 100 µL⁻¹ streptomycin/penicillin (Gibco, Life Technologies). In the case of NIH-3T3, 300,000 cells were plated on 6-well plates. Next, the cells were washed twice with phosphate buffer solution (PBS) and serum starved overnight. When indicated, cells were transfected overnight with 1 µg of cDNA. ATX (20 nM) was incubated with inhibitors (5 µM), LPC (1 µM) or LPA (1 µM), for 30 min in serum-free medium containing 0.05% (w/v) FAF-BSA (total volume 1 mL). Medium from the 12-well plates was removed and replaced with 1 mL of the ATX–inhibitor mixture. All cell lines were stimulated for 5 min (unless otherwise stated), after which the medium was removed and the plates immediately frozen on dry ice and stored at –80°C. For Western blotting, cells were washed with cold PBS, lysed in RIPA buffer, supplemented with protease inhibitors and protein phosphatase inhibitors (20 mM NaF and 1 mM orthovanadate; Pierce), and centrifuged. Protein concentration was measured using a BCA protein assay kit (Pierce), after which LDS sample buffer (NuPAGE, Invitrogen) and 1 mM dithiothreitol (DTT) were added to the lysate. SDS-PAGE pre-cast gradient gels (4–12% NuPAGE Bis-Tris, Invitrogen) were loaded with 20 µg of total protein, followed by transfer to
nitrocellulose membrane. Non-specific protein binding was blocked by 5% BSA in PBS-Tween (0.1%); primary antibodies (D9L: phospho-AKT [Ser473], 1:1,000; 4370S: phospho-ERK1/2 [Thr202/Tyr204], 1:1,000; Cell Signaling Technology) were incubated overnight at 4°C in PBS-Tween with 5% BSA containing 0.1% NaN₃. Horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark) were incubated for 1 h (RT; 293 K) in PBS-Tween with 2.5% BSA and developed using ECL Western blot reagent.

**Rho GTPase biosensor**

A fluorescence resonance energy transfer (FRET) pair consisting of RhoA-Cerulean3 and PKN fused to circularly permuted Venus was used. The HR1 region of PKN was used as the effector domain for activated RhoA. Experiments were performed in phenol red-lacking DMEM medium at 37°C. Cells were allowed to adhere overnight on uncoated coverslips, after which they were serum starved and transfected with the biosensor for 24 h. Next, the coverslips were placed on a thermostat-controlled (37°C) inverted Nikon Diaphot microscope and excited at 425 nm. Cells were stimulated with LPC (1 μM), LPA (1 μM), ATX (20 nM) or inhibitors (5 μM), while donor and acceptor emission were detected simultaneously for 10 min with two photomultipliers, using a 505-nm beam splitter and optical filters: 470 ± 20 nm (CFP channel) and 530 ± 25 nm (YFP channel). The emission data were analyzed using the Fiji software and normalized to control cells. At least three independent experiments were analyzed for every condition (20 fields of view/condition, 3–5 cells/fi eld of view, >50 cells/condition). FRET was expressed as the ratio between acceptor and donor signals, set at 1 at the onset of the experiment.

**Cell migration assays**

Cell migration was measured using 48-well chemotaxis chambers (Neuro Probe, Inc.) equipped with 8 mm-pore polycarbonate membranes, coated with fibronectin (20 mg/mL). Cells (4.8 × 10⁵/mL) were added to the upper chamber. FAF-BSA (0.5 mg/mL) was used as a lysophospholipid carrier. As chemoattractants in the lower chamber, we used LPC (1 μM), LPA (1 μM), ATX (20 nM) or inhibitors (5 μM) (unless otherwise stated). For NIH3T3 cells, migration was allowed for 4 h at 37°C in humidified air containing 5% CO₂. Migrated cells were fixed in Diff-Quik Fix and stained using Diff-Quik II. Migration was quantified by color intensity measurements using Adobe Photoshop.

** Vesicle internalization**

Serum-starved LPA₁- and LPA₆-HA-expressing HeLa-Flp-In cells cultured on 24 mm (#1,5) were treated with LPA (1 μM) for 15 min in DMEM containing 0.05% fatty acid-free BSA. Subsequently, coverslips were washed and fixed with 4% PFA, permeabilised with 0.1% Triton X-100 and blocked with 2% BSA for 1 h. Incubation with anti-HA antibody (3F10 from Roche Diagnostics; 1:200) was done for 1 h, followed by incubation with donkey anti-rat Alexa Fluor 488-conjugated antibody (A-21208 from Invitrogen; 1:200) for 1 h at room temperature. For confocal microscopy, cells were washed with PBS, mounted with Immmuno-MountTM (Thermo Scientific), visualized on a LEICA TCS-SP5 confocal microscopy (63x objective) and analyzed using ImageJ software.

**Production of LPAR-expressing HeLa-Flp-In cells**

cDNA containing human LPAR1, LPAR2, LPAR3, and LPAR6 was amplified by PCR to remove its stop codon and add the restriction sites for BamHI and XhoI (or NotI for LPAR6), after which it was subcloned in an in-house produced pDNAs.1-HA vector. Codon-optimized gene blocks for LPAR4 and LPAR5 were ordered to facilitate amplification and cloning strategies, which included the addition of restriction sites for BamHI and XhoI (or NotI for LPAR5). For plasma membrane localization, and based on previously produced unpublished data, the signal peptide of human neuronal acetylcholine receptor subunit alpha-7 (CHRNA7) (Gene ID: 1139; UniprotKB identifier: P36544) was added at the N-terminus of P2Y LPARs. This was done to avoid the predominant localization of wildtype P2Y LPARs at the nucleus and endoplasmic reticulum when overexpressed in HeLa Cells. 0.2 μg of the resulting vectors and 1.8 μg pOG44 Flp-Recombinase Expression Vector (Invitrogen) were incubated with 6 μL FuGENE 6 (Invitrogen) in 200 μL Opti-MEM (Gibco) for 30 min, after which the mix was added to previously produced HeLa-Flp-In cells. Cell medium was refreshed 24 h later, and selection with 1 μg/mL puromycin was started and maintained with resistant cells.

**Real-time quantitative PCR**

Cells were allowed to grow to almost complete confluency on 10-cm dishes in 10% FCS-containing DMEM. Total RNA was extracted using the GeneJET purification kit (Fermentas). cDNA was synthesized by reverse transcription from 5 μg RNA using First Strand cDNA Synthesis Kit (Thermo Scientific). RTqPCR was performed on a 7500 Fast System (Applied Biosystems) as follows: 95°C for 2 min, 95°C for 0 min, 60 cycles at 95°C for 15 s, followed by 60°C for 1 min for annealing and extension. The final reaction mixtures (12 μL) consisted of diluted cDNA, 16SYBR Green Supermix (Applied Biosystems), 200 nM forward primer, and 200 nM reverse primer. Reactions were performed in 384-well plates, with three independent biological replicas. As a negative control, the cDNA was replaced by MilliQ water. Cyclophilin was used as reference gene. Each sample was analyzed in triplicate and the normalized expression (NE) data were calculated using the equation $\text{NE} = 2^{-(Ct \text{target} - Ct \text{reference})}$. 

**MD simulations**

Models of human wildtype autotaxin (Uniprot code Q13822) and mutants T210A, G257K and W255A in complex with 18:1 LPA were constructed on the basis of our previously reported crystal structures of rat autotaxin (PDB codes 5DLW and 5DLT) as templates (94% sequence identity) as previously described. All MD simulations were performed using the GROMACS software version...
Each ATX (wildtype or mutant) - 18:1 LPA molecule complex contained 10 extra 18:1 LPA molecules at random positions with a minimum distance of 40 Å to the tunnel, ~58,500 water molecules, and Na+ ions (for total charge equilibrium) in a 12.5 x 12.5 x 12.5-nm simulation box (total number of ~189,000 atoms), and was minimized and equilibrated until 10 independent 150 ns MD trajectories were produced in a process we have described previously.18 For each of the 10 MD simulation, the trajectories of all 10 LPA molecules were followed, resulting in 100 trajectories indicated in the graphs of Figure 3C. The total simulation time for the four complexes and replicas was added up to a total of 6.0 μs.

Animal studies

Detection of rat LPA plasma levels

Whole blood was collected from rats in sodium heparin tubes. The samples were centrifuged at 3,000 rpm for 15 min at 4°C to separate the plasma, which was stored at −80°C. CpdA and ziritaxestat were serially diluted in dimethyl sulfoxide (DMSO) and 0.5 μL of the dilutions dispensed into 96-well plates placed on ice; plasma was thawed on ice. Next, 49.5 μL of plasma were added to wells containing 0.5 μL of ziritaxestat or CpdA (1% final DMSO concentration). The plates were covered with a polypropylene lid and incubated for 2 h at 37°C in humidified air containing 5% CO2, with gentle shaking (except for the control samples, which were stored at −20°C). The control samples were thawed on ice, then transferred to the incubated plates before liquid chromatography–mass spectrometry analysis. For the analysis, 10 μL of plasma proteins from the incubated plates were precipitated with an excess of methanol containing the internal standard 17:0 LPA (Cat# 857127, Avanti Polar Lipids, Inc.). The samples were centrifuged and the supernatants injected onto a C18 column. Analytes were eluted off the column under isocratic conditions. An API5500 QTRAP mass spectrometer (ABSciex™) was used for the detection of 18:2 LPA. Relative quantities were calculated based on the peak area.

Radiation-induced fibrosis mouse model

Procedure and drug-naïve seven-week-old female B6 albino (B6N-Tyrc−BrdCrCrl) mice (16–18 g) from Charles River (France) were delivered at the Plateforme de Radiothérapie Expérimentale in Institut Curie (Orsay, France). After a 7-day acclimatization period, they were identified using the micro-tattoo Aramis system. Mice were anesthetized with isoflurane, placed in holders, and irradiated with a single 17-Gy dose in the thorax area (Week 1). All mice survived after irradiation. The week after irradiation, mice were shipped to Galapagos Animal Facilities (Romainville, France), where they were housed 10 per cage after individual identification using micro-chip (Biolog-id, Boulogne-Billancourt, France). They were maintained at 22°C on a 12-h light/dark cycle (07:00–19:00); food and water were provided ad libitum. The study was performed according to the Animal Institutional Care and Use Committee of Galapagos, ethical guidelines of animal experimentation, and the guidelines for welfare of animals in experimentation. At Week 13, fur was removed on the thorax of the anesthetized mice (using a shaving razor, then hair removing cream). In vivo imaging using a proprietary collagen near-infrared fluorescent probe was used to exclude mice presenting liver fibrosis (~15%), and to allocate them to each experimental group according to their lung fibrosis level. Treatment with ziritaxestat (30 mg/kg b.i.d) or CpdA (10 mg/kg b.i.d) was assayed by oral route, in comparison to nintedanib (60 mg/kg q.d). The dose of ziritaxestat (30 mg/kg) was selected based on previous experiments in the bleomycin-induced lung fibrosis model, where a dose of 30 mg/kg showed activity, coinciding with the dose employed in clinical trials. Treatment was initiated 13 weeks after irradiation and lasted for 6 weeks. On Week 17, 4 weeks after initiation of dosing, steady-state pharmacokinetics were assessed, with sampling at four time points: 0, 1, 3, and 6 h (assuming 24 h is t = 0 h). Plasma was prepared and kept at −20°C until quantification using liquid chromatography–mass spectrometry. At Week 19, mice were killed by elongation. Entire lungs were removed, rinsed with PBS, and weighed. Lobes of the lungs were separated and fixed in formaldehyde for 48 h before paraffin embedding. For every lung, 4-μm thick sections were immunostained with anti-collagen I antibody. The immunohistochemistry samples were processed automatically using the immunostainer BOND-RX (Leica). The stained sections were scanned (NanoZoomer, Hamamatsu Photonics K.K.) before quantification by image analysis (CaloPix, Tribun Health). Data were expressed as percentage of collagen I area per area of lung tissue devoid of the main constitutive collagen in vessels and bronchi.

Organic chemistry methods

Structure of GLPG1690 and CpdA
Synthesis of ziritaxestat

GLPG1690/ziritaxestat synthesis was published in.25 Ziritaxestat was synthetized in five steps as depicted below.

**Synthesis of int A**

\[
\begin{align*}
N-(6\text{-Bromo-2-ethyl-8-methyl-imidazo}[1,2-a]pyridin-3-yl)formamide \text{ (Int A)} \quad &\xrightarrow{(a)} \quad \text{To a suspension of 2-amino-5-bromo-3-methylpyridine (420 g, 2.24 mol, washed before use with an aqueous saturated NaHCO}_3\text{ solution) in toluene (1.5 L) under nitrogen atmosphere were added propanal (248 mL, 3.36 mol) and benzotriazole (281 g, 2.36 mol). The resulting mixture was stirred 4 h at room temperature. Then ethanol (3.5 L) and potassium cyanide (175 g, 2.70 mol) were added. CAUTION! Potassium cyanide is highly toxic. The reaction mixture was further stirred overnight at room temperature and refluxed for 2 h. After cooling to room temperature, the mixture was quenched by addition of a 2.5M NaOH aqueous solution (3 L). This experiment was performed in four batches with the same quantities of reagents, the crude mixtures were then pooled together and concentrated in vacuo to low volume. The remaining oil was diluted with EtOAc (15 L) and washed with a 2M NaOH aqueous solution (2 × 2 L). The aqueous layer was extracted twice with EtOAc (2 × 1 L). The combined organic layers were then dried over Na\textsubscript{2}SO\textsubscript{4}, filtered and concentrated in vacuo. The crude mixture was dissolved in ethanol (2 L) and carefully added to a solution of acetyl chloride (1 L, 14.0 mol, 1.6 eq.) in ethanol (6 L). The resulting reaction mixture was stirred at room temperature overnight and then concentrated to dryness. The residue was triturated in DCM (7 L) for 3 days, the precipitate formed was collected, washed with DCM (2 × 500 mL) and dried to afford 6-bromo-2-ethyl-8-methylimidazo[1,2-a]pyridin-3-amine as a hydrochloride salt (791 g, 29%).1\text{H NMR (400 MHz, DMSO-d}_6\text{), presence of 2 rotamers, }d(\text{ppm}) 10.2 \text{(br s, 1 H), 8.51 (s, 1 H, one rotamer), 8.36 (s, 1 H, one rotamer), 8.23 (s, 1 H, one rotamer), 8.11 (s, 1 H), 7.23 (s, 1 H, one rotamer), 2.63-2.60 (m, 2 H), 2.58 (s, 3 H, one rotamer), 2.56 (s, 3 H, one rotamer), 1.24-1.17 (m, 3 H). LC-MS: m/z = 282.0/284.0 \text{ [M}+\text{H}].
\end{align*}
\]

**Synthesis of int B**

\[
\begin{align*}
6\text{-Bromo-2-ethyl-N,8-dimethyl-imidazo}[1,2-a]pyridin-3-amine \text{ (Int B)} \quad &\xrightarrow{(a)} \quad \text{To a suspension of N-(6-bromo-2-ethyl-8-methyl-imidazo[1,2-a]pyridin-3-yl)formamide (78.2 g, 277 mmol) and potassium carbonate (114.8 g, 831 mmol) in acetone (923 mL), iodomethane (25.9 mL, 416 mmol) was added at room temperature and the reaction mixture was stirred at 80\textdegree C overnight. The solids were filtered off and rinsed with acetone and DCM. The filtrate was concentrated and the obtained solid was triturated in Et}_2\text{O, filtered and rinsed with Et}_2\text{O and water. The solid was collected and dried under vacuum to afford N-(6-bromo-2-ethyl-8-methyl-imidazo[1,2-a]pyridin-3-yl)formamide (724 g, 95%).1\text{H NMR (400 MHz, DMSO-d}_6\text{), presence of 2 rotamers, }d(\text{ppm}) 10.2 \text{(br s, 1 H), 8.51 (s, 1 H, one rotamer), 8.36 (s, 1 H, one rotamer), 8.23 (s, 1 H, one rotamer), 8.11 (s, 1 H), 7.23 (s, 1 H, one rotamer), 7.21 (s, 1 H, one rotamer), 2.63-2.60 (m, 2 H), 2.58 (s, 3 H, one rotamer), 2.56 (s, 3 H, one rotamer), 1.24-1.17 (m, 3 H). LC-MS: m/z = 282.0/284.0 \text{ [M}+\text{H}].
\end{align*}
\]
[1,2]-pyridin-3-yl]-N-methyl-formamide as a white solid (75.9 g, 93%). $^1$H NMR (400 MHz, CDCl$_3$), presence of 2 rotamers, \( \delta \) (ppm) 8.49 (s, 1H, minor rotamer), 8.19 (s, 1 H, major rotamer), 7.78 (s, 1H, major rotamer), 7.65 (s, 1H, minor rotamer), 7.15 (s, 1H, major rotamer), 7.08 (s, 1 H, minor rotamer), 3.36 (s, 3 H, minor rotamer), 3.24 (s, 3 H, major rotamer), 2.73-2.70 (m, 2 H), 2.59 (s, 3 H), 1.31 (t, \( J = 7.6 \) Hz, 3 H). LC-MS: m/z = 296.0/298.0 [M+H].

(b) A solution of HCl 1.25 M in methanol (35 mL, 43.8 mmol) was added to N-(6-bromo-2-ethyl-8-methyl-imidazo[1,2-a]pyridin-3-yl]-N-methyl-formamide (5.0 g, 16.9 mmol) and the reaction mixture was stirred for 4 h at 80°C (oil bath temperature). Another addition of HCl 1.25 M in methanol (14 mL, 17.5 mmol) was performed and the reaction mixture was stirred at 80°C for 1 h then at 100°C overnight. Solvent was evaporated and the crude product was partitioned between water and DCM. The aqueous layer was basified with NaHCO$_3$ and extracted with DCM. Combined organic layers were washed with brine, dried over sodium sulfate, filtered and evaporated to give 6-bromo-2-ethyl-N,8-dimethyl-imidazo[1,2-a]pyridin-3-amine (Int B) (4.44 g, 98%) that was used in the next step without further purification. $^1$H NMR (400 MHz, CDCl$_3$) \( \delta \) ppm 8.05 (s, 1 H), 7.04 (s, 1 H), 2.84-2.78 (m, 5 H), 2.60 (s, 3 H), 1.35 (t, \( J = 7.6 \) Hz, 3 H). LC-MS: m/z = 268.2/270.3 [M+H].

**Synthesis of int C**

2-(6-Bromo-2-ethyl-8-methyl-imidazo[1,2-a]pyridin-3-yl)-methyl-amino]-4-(4-fluorophenyl)thiazole-5-carbonitrile (Int C). To a solution compound Int B (4.4 g, 16.6 mmol) in THF (44 mL) under argon was slowly added NaH (60% in oil suspension, 2.0 g, 50.0 mmol). The reaction mixture was heated at 90°C (oil bath temperature) for 30 min then cooled to 40°C before adding 2-chloro-4-(4-fluorophenyl)thiazole-5-carbonitrile (4.74 g, 19.9 mmol). The reaction mixture was stirred at 90°C overnight. After cooling to room temperature the mixture was slowly quenched by addition of water and then diluted with ETOAc. The organic layer was separated and the aqueous layer extracted with ETOAc. The organic layers were then washed with water and brine, filtered, and evaporated to afford int C as an orange solid (5.7 g, 73%). $^1$H NMR (400 MHz, CDCl$_3$) \( \delta \) ppm 8.21-8.15 (m, 2 H), 7.82 (s, 1 H), 7.25-7.18 (m, 3 H), 3.66 (s, 3 H), 2.85-2.76 (m, 2 H), 2.68 (s, 3 H), 1.38 (t, \( J = 7.6 \) Hz, 3 H). LC-MS: m/z = 470.3/472.3 [M+H].

**Synthesis of int D**

2-(6-Bromo-2-ethyl-8-methyl-imidazo[1,2-a]pyridin-3-yl)-methyl-amino]-4-(4-fluorophenyl)thiazole-5-carbonitrile (Int C). To a solution compound Int B (4.4 g, 16.6 mmol) in THF (44 mL) under argon was slowly added NaH (60% in oil suspension, 2.0 g, 50.0 mmol). The reaction mixture was heated at 90°C (oil bath temperature) for 30 min then cooled to 40°C before adding 2-chloro-4-(4-fluorophenyl)thiazole-5-carbonitrile (4.74 g, 19.9 mmol). The reaction mixture was stirred at 90°C overnight. After cooling to room temperature the mixture was slowly quenched by addition of water and then diluted with ETOAc. The organic layer was separated and the aqueous layer extracted with ETOAc. The organic layers were then washed with water and brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was triturated in Et$_2$O, filtered and washed with Et$_2$O and MeCN. Recrystallization was performed in MeCN (180 mL) to afford compound Int C as an orange solid (5.7 g, 73%). $^1$H NMR (400 MHz, CDCl$_3$) \( \delta \) ppm 8.21-8.15 (m, 2 H), 7.82 (s, 1 H), 7.25-7.18 (m, 3 H), 3.66 (s, 3 H), 2.85-2.76 (m, 2 H), 2.68 (s, 3 H), 1.38 (t, \( J = 7.6 \) Hz, 3 H). LC-MS: m/z = 470.3/472.3 [M+H].
2-[(2-Ethyl-8-methyl-6-piperazin-1-yl-imidazo[1,2-a]pyridin-3-yl)-methyl-amino]-4-(4-fluorophenyl)thiazole-5-carbonitrile (Int D). (a) To a solution of compound Int C (24.2 g, 51.5 mmol) in toluene under argon were successively added N-boc-piperazine (14.4 g, 77.3 mmol), sodium tert-butoxide (9.9 g, 103 mmol), JohnPhos (1.54 g, 5.15 mmol) and Pd2(dba)3 (2.36 g, 2.58 mmol). The reaction mixture was heated at 115°C for 1 h. After cooling to room temperature, the reaction was filtered on Celpure® P65 and the filtrate was evaporated. The residue was dissolved in EtOAc and washed with water. The organic layer was further washed with brine, dried over Na2SO4, filtered and concentrated in vacuo. The crude product was purified by chromatography on silica gel to afford tert-butyl 4-[3-[[5-cyano-4-(4-fluorophenyl)thiazol-2-yl]-methyl-amino]-2-ethyl-8-methyl-imidazo[1,2-a]pyridin-6-yl]piperazine-1-carboxylate (24.4 g, 82%). 1H NMR (400 MHz, CDCl3) δ (ppm) 8.18-8.14 (m, 2 H), 7.21-7.16 (m, 2 H), 7.08-7.02 (m, 2 H), 3.62 (s, 3 H), 3.61-3.57 (m, 4H), 3.07-2.96 (m, 4H), 2.80 (q, J = 7.6 Hz, 2 H), 2.66 (s, 3 H), 1.47 (s, 9 H), 1.36 (t, J = 7.6 Hz, 3 H). LC-MS: m/z = 576.6 [M+H].

(b) To a solution of the latter compound (24.4 g, 42.4 mmol) in MeOH (100 mL) was added a 2 M HCl solution in Et2O (127 mL, 254 mmol). The reaction mixture was stirred at room temperature for 3.5 h then concentrated in vacuo. The residue was partitioned between EtOAc and water. The aqueous layer was extracted twice with EtOAc. A 2 M NaOH solution was added to the aqueous layer until pH 8-9 was reached and further extraction with EtOAc was performed. The combined organic layers were then washed with brine, dried over Na2SO4, filtered and concentrated in vacuo. The solid was stirred in heptane (100 mL) at room temperature overnight, filtered off, washed with heptane and Et2O, and dried to afford 2-[(2-ethyl-8-methyl-6-piperazin-1-yl-imidazo[1,2-a]pyridin-3-yl)-methyl-amino]-4-(4-fluorophenyl)thiazole-5-carbonitrile (Int D) (18.06 g, 90%). 1H NMR (400 MHz, CDCl3) δ (ppm) 8.23-8.16 (m, 2 H), 7.24-7.16 (m, 2 H), 7.06-7.00 (m, 2 H), 3.61 (s, 3 H), 3.09-2.98 (m, 8 H), 2.75 (q, J = 7.6 Hz, 2 H), 2.61 (s, 3 H), 1.34 (t, J = 7.6 Hz, 3 H). LC-MS: m/z = 476.5 [M+H].

Synthesis of ziritaxestat

2-[(2-Ethyl-6-[4-[2-(3-hydroxyazetidin-1-yl)-2-oxo-ethyl]piperazin-1-yl]-8-methyl-imidazo[1,2-a]pyridin-3-yl]-methyl-amino]-4-(4-fluorophenyl)thiazole-5-carbonitrile (ziritaxestat). To a solution of 2-[(2-ethyl-8-methyl-6-piperazin-1-yl-imidazo[1,2-a]pyridin-3-yl)-methyl-amino]-4-(4-fluorophenyl)thiazole-5-carbonitrile (Int D) (18.05 g, 38 mmol) in MeCN (126 mL) were added potassium carbonate (10.49 g, 75.9 mmol) and 2-chloro-1-(3-hydroxyazetidin-1-yl)ethanone (7.38 g, 49.3 mmol). The reaction mixture was refluxed for 3.5 h then filtered and the solid was washed with MTBE and MeCN. The collected precipitate was then suspended in 300 mL of water, stirred for 1 h, filtered, and finally washed with water. The solid obtained was dried in vacuo to afford 2-[(2-ethyl-6-[4-[2-(3-hydroxyazetidin-1-yl)-2-oxo-ethyl]piperazin-1-yl]-8-methyl-imidazo[1,2-a]pyridin-3-yl]-methyl-amino]-4-(4-fluorophenyl)thiazole-5-carbonitrile (ziritaxestat) (18.0 g, 81%). 1H NMR (400 MHz, CDCl3) δ ppm 8.20-8.12 (m, 2 H), 7.22-7.13 (m, 2 H), 6.99 (s, 2 H), 4.75-4.66 (m, 1 H), 4.51-4.43 (m, 1 H), 4.34-4.26 (m, 1 H), 4.14-4.05 (m, 1 H), 3.88 (dd, J = 10.9, 4.4 Hz, 1 H), 3.61 (s, 3 H), 3.14-3.02 (m, 6 H), 2.74 (q, J = 7.7 Hz, 2 H), 2.70-2.62 (m, 4 H), 2.59 (s, 3 H), 1.33 (tt, J = 7.6 Hz, 3 H). LC-MS: m/z = 589.6 [M+H].

Synthesis of CpdA

CpdA was synthetized in five steps as depicted below.

Synthesis of Int 1
tert-Butyl 2-amino-5h-pyrrolo-[3,4-d]pyrimidine-6(7h)-carboxylate (11 g, 46.6 mmol, 1.0 eq.), 1-bromo-3,5-dimethyl-benzene (9 g, 48.9 mmol, 1.05 eq.), Xantphos (5.49 g, 9.31 mmol, 0.2 eq.), NaOtBu (13.8 g, 139.7 mmol, 3.0 eq.), and Pd2(dba)3 (4.35 g, 4.7 mmol, 0.1 eq.) were placed in 1,4-dioxane (150 mL) under Ar atmosphere and the mixture was vigorously stirred at 110 °C for 3 h then at 80 °C overnight. The reaction medium was poured into a well-stirred mixture of NaCl (250 g) in EtOAc/water (2/1, 1.5 L). The layers were separated, and the aqueous phase was extracted with EtOAc. The combined organic layers were filtered over Celite®, dried over Na2SO4, filtered, and concentrated. The residue was suspended in an EtOAc/cyclohexane mixture (1/1, 200 mL) and the resulting precipitate was filtered and washed with an EtOAc/cyclohexane mixture (1/1). The solid was dried to afford Int 1.

**Synthesis of Int 2**

\[ \text{Int 1} \] (39.8 g, 1151.7 mmol, 1.0 eq.) was suspended in a solution of 4N HCl in 1,4-dioxane (400 mL). The mixture was stirred at RT for 3 h, then concentrated to dryness. The resulting solid was dissolved in water, and DCM and 2N NaOH were added. The layers were separated and the aqueous layer was back-extracted twice with DCM. The organic layers were evaporated to dryness to obtain Int 2.

**Synthesis of Int 3**

\[ \text{Int 2} \] (27 g, 112.4 mmol, 1.0 eq.) and tert-butyl (2R)-2-methyl-4-oxo-piperidine-1-carboxylate (CAS# 790667-43-5; 25.2 g, 118.2 mmol, 1.05 eq.) were stirred in DCM (350 mL) and AcOH (0.7 mL) for 30 min under inert atmosphere. NaBH(OAc)3 (35.6 g, 168.0 mmol, 1.5 eq.) was added and the mixture was stirred at RT for 1 h. The mixture was poured in a NaOAc solution before the aqueous phase was extracted with DCM, dried over Na2SO4, and concentrated to dryness. The crude product was purified by flash column chromatography on silica gel (eluted with heptane/EtOAc) to afford Int 3.

**Synthesis of Int 4**

\[ \text{Int 3} \] (15.4 g, 35.2 mmol, 1.0 eq.) was suspended in HCl (4N in 1,4-dioxane, 250 mL) and the mixture was stirred at RT for 2 h. The medium was concentrated to dryness and the residue was taken up in DCM. Water was added and the pH of the aqueous phase was adjusted to 10 using 40% NaOH aq. solution. The layers were separated, and the aqueous layer was extracted with DCM. The combined organic phases were concentrated to afford Int 4.

**Synthesis of CpdA**

\[ \text{Cpd A} \]
6-fluoro-1H-benzotriazole-5-carboxylic acid (CAS# 1427081-62-6; 5.4 g, 29.8 mmol, 1.0 eq.), HATU (11.4 g, 29.8 mmol, 1.0 eq.), HOAt (2.0 g, 14.9 mmol, 0.5 eq.), and tetramethyl piperidine (10.5 g, 74.5 mmol, 2.5 eq.) were dissolved in DMF (100 mL). The reaction mixture was stirred at RT for 5 min. Int 4 (10.1 g, 30.0 mmol, 1.03 eq.) was then added and the mixture was stirred at RT overnight. The reaction mixture was poured on a sat. NH₄Cl aq. solution (400 mL) and stirred for 40 min in an ice bath. The precipitate was filtered off, rinsed with water, dried under suction for 1 h and under vacuum at 40 °C to yield crude material. The crude product was purified by chromatography on silica gel, eluting with DCM:MeOH:NH₄OH (90:9:1.5). The fractions of interest were pooled and concentrated under reduced pressure. The solid material obtained was triturated in acetone (20 vol.) at 0 °C for 40 min, filtered, and dried under vacuum for 4 h to afford CpdA.

**UPLC/MS chromatogram of ziritaxestat**

Analysis was performed on a Waters Acquity UPLC with Waters Acquity PDA detector and SQD mass spectrometer, and equipped with an Acquity UPLC BEH C18 1.7μm 2.1 × 5mm VanGuard pre-column and an Acquity UPLC BEH C18 1.7μm 2.1 × 50mm column, using a water/acetonitrile gradient containing 0.05% concentrated ammonia solution at a flow rate of 1 mL/min.

![UPLC/MS chromatogram of ziritaxestat](image-url)
LC chromatogram and Mass spectrum of ziritaxestat

NMR spectra of ziritaxestat

Ziritaxestat was assessed by $^1$H-NMR and $^{13}$C-NMR in CDCl$_3$ and found to be consistent with the structure.
$^1$H NMR spectrum of ziritaxestat in CDCl$_3$

$^{13}$C NMR spectrum of ziritaxestat in CDCl$_3$

**UPLC/MS chromatogram of CpdA**

Analysis was performed on a Waters Acquity UPLC with Waters Acquity PDA detector and SQD mass spectrometer, and equipped with an Acquity UPLC BEH C18 1.7µm 2.1 × 5mm VanGuard pre-column and an Acquity UPLC BEH C18 1.7µm 2.1 × 50mm column, using a water/acetonitrile gradient containing 0.05% concentrated ammonia solution at a flow rate of 1 mL/min.
**NMR spectra of CpdA**

$^1$H NMR (400 MHz, CD$_3$OD) δ 8.25 (s, 1H), 8.17-7.88 (m, 1H), 7.82-7.60 (m, 1H), 7.30-7.18 (m, 2H), 6.79-6.61 (m, 1H), 4.89-4.72 (m, 1H), 4.03-3.81 (m, 4H), 3.72-3.46 (m, 2H), 3.07-2.73 (m, 1H), 2.30 (s, 6H), 2.19-1.72 (m, 4H), 1.63-1.41 (m, 3H).
QUANTIFICATION AND STATISTICAL ANALYSIS

All data are expressed as the average value of triplicate biological measures ± standard error of the mean (S.E.M.). GraphPad Prism 9.0.2 was used to analyze the significance between sample groups. Statistical analyses of samples versus negative controls were performed using one-way ANOVA with Dunnett’s post-hoc test. All other statistical comparisons were performed using one-way ANOVA with Tukey’s post-hoc test. In all cases, significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.