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# Rational design of Autotaxin inhibitors by structural evolution of endogenous modulators

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## Abstract

Autotaxin produces the bioactive lipid lysophosphatidic acid (LPA), and is a drug target of considerable interest for numerous pathologies. We report the expedient, structure-guided evolution of weak physiological allosteric inhibitors (bile salts) into potent competitive Autotaxin inhibitors that do not interact with the catalytic site. Functional data confirms that our lead compound attenuates LPA mediated signalling in cells, and reduces LPA synthesis *in vivo*, providing a promising natural product derived scaffold for drug discovery.

## Introduction

Autotaxin (ATX, ENPP2) is responsible for the production of the bioactive lipid lysophosphatidic acid (LPA) from lysophospholipids which exist extracellularly, primarily derived from lysophosphatidylcholine (LPC).<sup>1</sup> The product of this process, LPA, is an agonist of six G protein-coupled receptors that drives a range of signaling pathways.<sup>2</sup> This action produces a broad range of biological outcomes, and the pathophysiology of ATX has been associated with pulmonary fibrosis, tumor progression, cardiovascular disease, neuropathic pain, and cholestatic pruritus; these functions make the ATX-LPA signaling pathway an attractive prospect from the point of view of therapeutic intervention.<sup>3</sup>

The catalytic domain of the enzyme consists of an active site containing two zinc ions in proximity to a catalytic threonine. The endogenous ligand (LPC) binds in a shallow hydrophilic groove that is capable of accommodating both nucleotides as well as the glycerol motif of lysophospholipids. There also exists a relatively deep hydrophobic pocket which is responsible for binding of the lysophospholipid acyl chain. The catalytic domain in conjunction with the first of two somatomedin- $\beta$  (SMB) domains, constitutes a tunnel adjacent to the active site.<sup>4-5</sup> Recently, we reported that ATX produced in mammalian cells contains steroids bound within the tunnel region. During that study, we determined the structures of natural endogenous steroids bound to the ATX tunnel, including that of tauroursodeoxycholic acid (TUDCA, **1**, Figure 1), a micromolar inhibitor of LPA production. We demonstrated that binding of bile salts, allosterically modulates ATX activity through non-competitive inhibition, likely having physiologically important implications for the function of the ATX-mediated signaling through LPA receptors in a number of pathophysiological contexts.<sup>6</sup>

The majority of small molecule inhibitors of ATX developed to date emulate the lipid-like structure of the native ligand and bind in the orthosteric site (e.g. **3**, Figure 1). By contrast, a recent series of indole-based ATX inhibitors<sup>7</sup> and the lead compound of an independent discovery program<sup>8</sup> are reported to occupy the hydrophobic pocket and tunnel, without

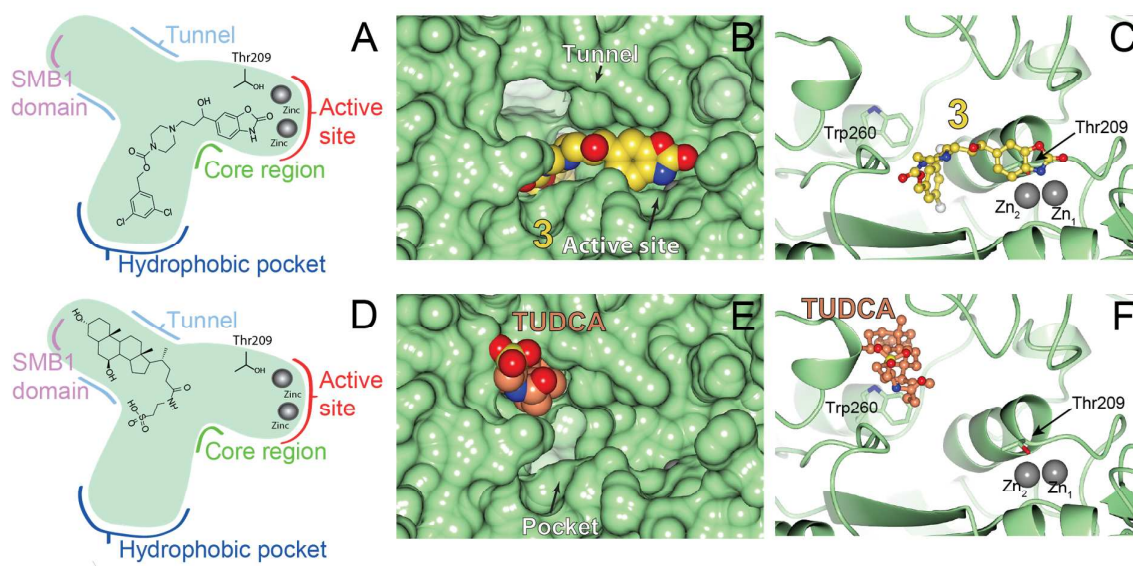
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3 interacting with the hydrophilic groove or the active site, acting as allosteric inhibitors, similar  
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5 to natural bile salts.  
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8 In this manuscript, we formulate a design hypothesis based on structural knowledge of the  
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10 binding mode of the partial non-competitive allosteric inhibitor bile salts, and competitive  
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12 orthosteric inhibitors.<sup>9</sup> Specifically, we used the structure of compound **1** bound to ATX  
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14 (Figure 1) which we recently determined binding in the allosteric tunnel region,<sup>6</sup> and the  
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16 structure of a compound from the represented by compound **2** (PF-8380),<sup>10</sup> which we had  
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18 determined in complex with ATX, showing the binding of this chemotype in the orthosteric  
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20 site.  
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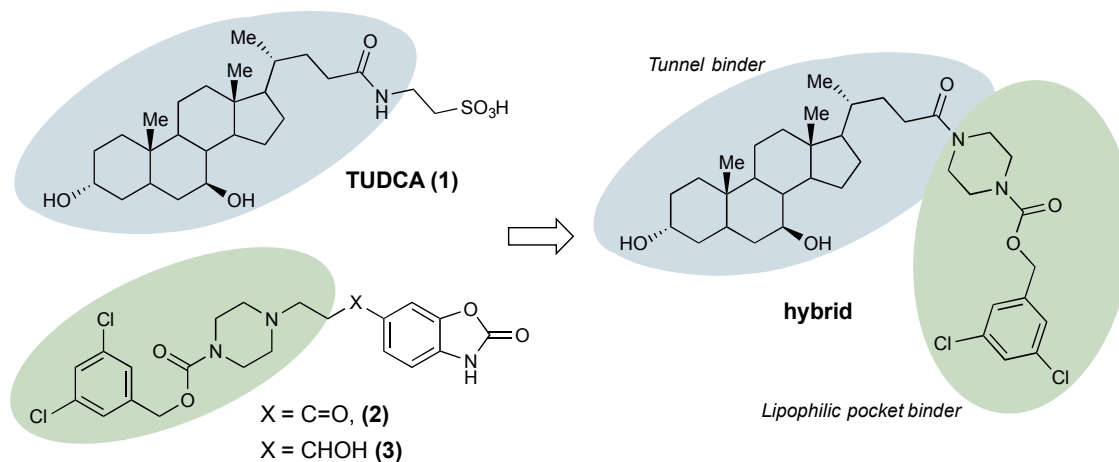
22 From consideration of these binding modes we inferred that the tunnel-binding steroid  
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24 skeleton could be merged with the lipophilic pocket-binding “tail” of compound **2**. While the  
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26 “tail” of compound **2** is in isolation ineffective,<sup>11</sup> as is the steroid group alone,<sup>6</sup> we reasoned  
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28 the hybridized structures may be potent inhibitors of ATX.  
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## Results and Discussion

We crystallized an alcohol analogue (**3**, Figure 2) of compound **2**, which, owing to its significantly higher aqueous solubility, proved more amenable to co-crystallization compared to the parent compound, **2**. We determined the crystal structure of **3** bound to ATX (Figure 1 A-C) to 2.1 Å resolution, showing that the 3,5-dichlorobenzyl moiety of **3** binds in the lipid binding pocket, consistent with a recent report of the binding mode of this series.<sup>12</sup> Superposing this structure of **3**, with that of **1** in complex with ATX<sup>6</sup> (Figure 1 D-F) enabled the design of a highly chemically tractable hybrid series through the union of the two binding motifs (Figure 2).

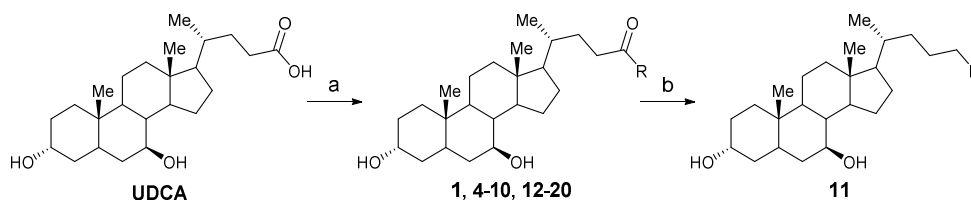


**Figure 1.** Crystal structure of **3** and **1** binding to the hydrophobic pocket and the tunnel of ATX, respectively. (A-C) show that **3** binds to the hydrophobic pocket. Compound **3** is racemic and the (*R*) enantiomer is resolved through co-crystallography; (D-F) show that **1** binds to the tunnel.

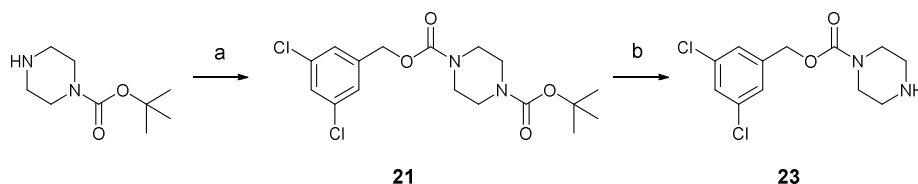


**Figure 2.** Overarching design hypothesis

These compounds could be rapidly prepared through HATU-mediated amide bond formation between ursodeoxycholic acid (UDCA) and appropriate amine fragments (Scheme 1). Compound **11** was accessed through reduction of the amide to give the corresponding amine derivative. The requisite amine components could be prepared through carbamate formation on the mono-protected diamine, followed by deprotection to furnish the free amine as represented by the example shown in Scheme 2.



**Scheme 1.** Conditions (a) amine (0.9 equiv), HATU (1.0 equiv), DIPEA (4.0 equiv), DMF, rt; (b)  $\text{BH}_3$ ·THF (10 equiv), THF, 60 °C.

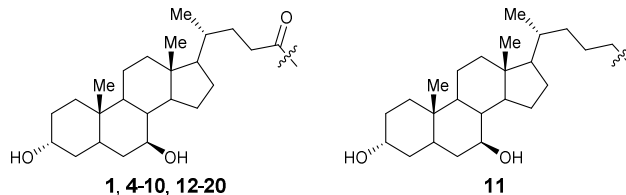


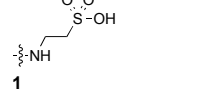
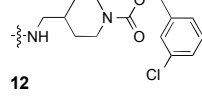
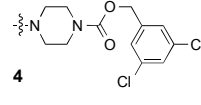
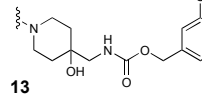
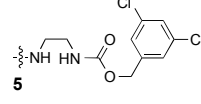
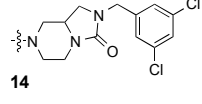
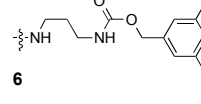
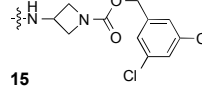
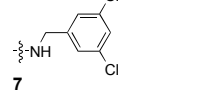
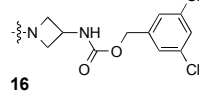
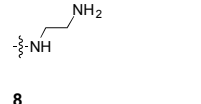
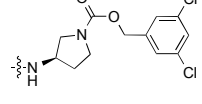
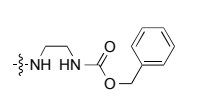
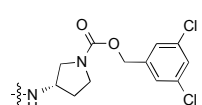
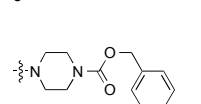
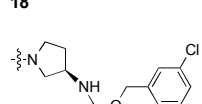
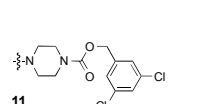
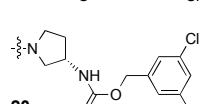
**Scheme 2.** (a) 1,1'-carbonyldiimidazole (1.1 equiv), 3,5-dichlorobenzylalcohol (1.1 equiv), amine (1.0 equiv), DMF, rt; (b) Trifluoroacetic acid/ $\text{CH}_2\text{Cl}_2$  (1:1).

To our delight, the first hybrid compound prepared (**4**, Table 1) showed approximately an 80-fold increase in potency relative to the progenitor bile acid (0.129  $\mu$ M compared to 10  $\mu$ M, determined through measurement of inhibition of LPC hydrolysis), confirming that the concept of maintaining the steroid moiety while also targeting the hydrophobic pocket of ATX had indeed increased inhibitor potency. In agreement with this hypothesis, compounds without chlorine substituents (**9-10**) or lacking a hydrophobic anchor entirely (**8**) in this initial set of analogues, results in no detectable inhibitory activity. These findings are consistent with SAR previously established for the hydrophobic pocket, all of which indicate a degree of lipophilicity in this region is required for adequate levels of ATX-inhibitory activity.<sup>10-13</sup>

In this initial survey of SAR, we also sought to examine the length and nature of the linker (**5-7**). While the piperazine unit in **4** was optimal, linear systems exhibit only slightly lower potency (**5-6**); however, a very short spacer (**7**) resulted in no detectable inhibitory activity. Compound **11**, lacking the amide carbonyl of **4**, had five-fold lower potency.

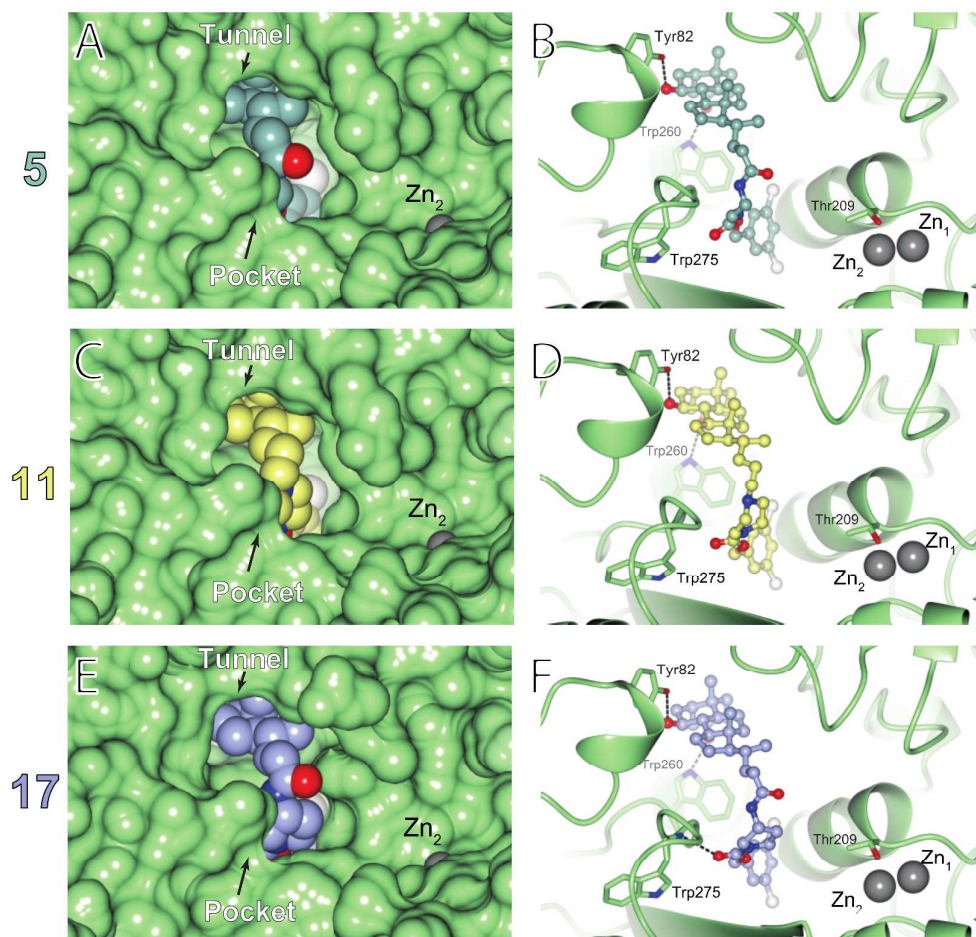
**Table 1.** Evaluation of hybrid compounds.



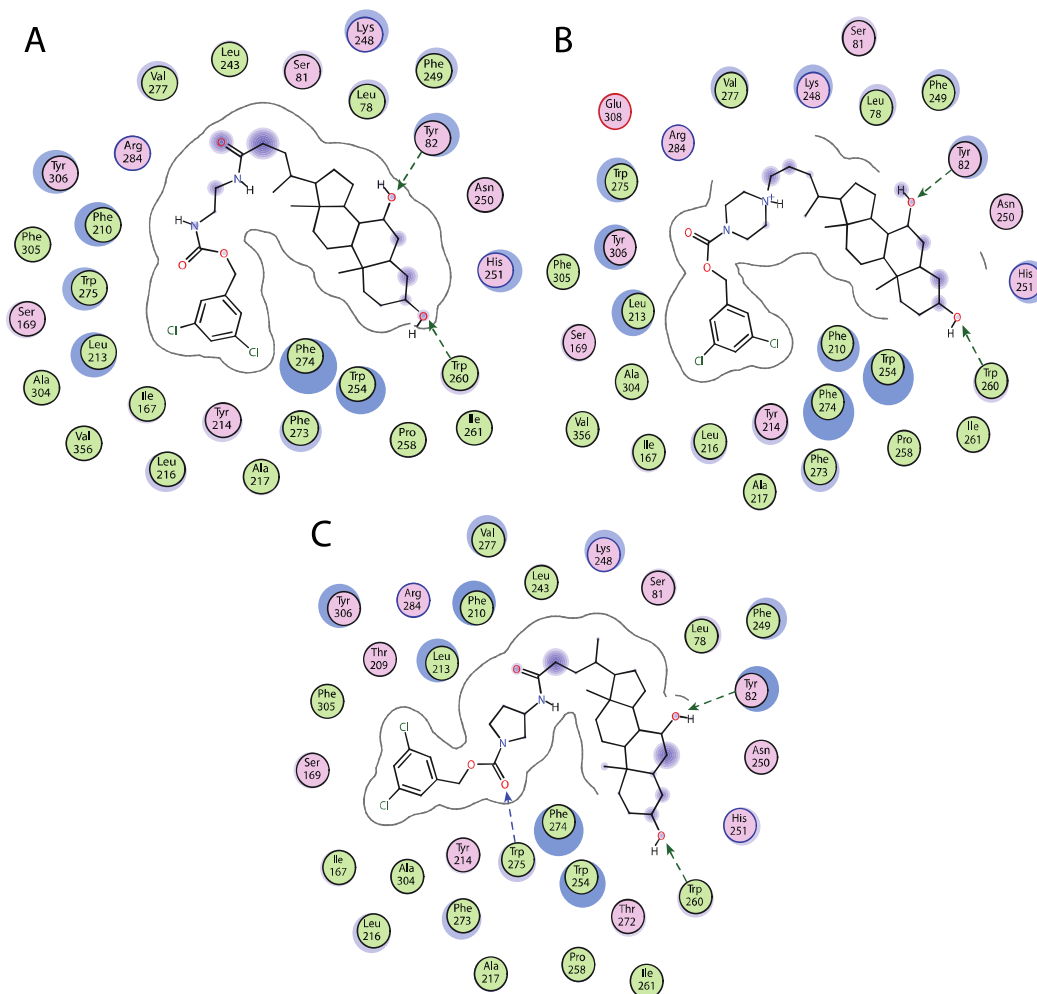
| Compound  | IC <sub>50</sub><br>(μM) | MW  | LogD <sup>b</sup> | Compound   | IC <sub>50</sub><br>(μM) | MW  | LogD <sup>b</sup> |
|---|--------------------------|-----|-------------------|--|--------------------------|-----|-------------------|
|    | 10.3                     | 500 | -0.2              |    | 2.5                      | 692 | 6.4               |
|    | 0.129                    | 664 | 6.0               |    | 0.100                    | 708 | 5.2               |
|    | 0.202                    | 638 | 5.7               |    | 2.10 <sup>a</sup>        | 675 | 5.4               |
|   | 0.563                    | 652 | 5.8               |   | 1.00                     | 650 | 5.8               |
|  | > 30                     | 551 | 6.1               |  | 0.208                    | 650 | 5.8               |
|  | > 30                     | 435 | 0.6               |  | 0.020                    | 664 | 5.9               |
|  | > 30                     | 569 | 4.5               |  | 0.717                    | 664 | 5.9               |
|  | > 30                     | 595 | 4.8               |  | 0.313                    | 664 | 5.9               |
|  | 0.814                    | 650 | 6.6               |  | 0.500                    | 664 | 5.9               |

[a] Epimeric mixture; [b] calculated at pH 7.4 using JChem for Excel.<sup>14</sup>





**Figure 3.** Crystal structure of ATX bound to **5**, **11** and **17**. The view and representation is similar to Figure 1.



**Figure 4.** Two-dimensional projection depicting the interactions between the three ligands **5** (A), **11** (B), **17** (C) and ATX, highlighting hydrogen bonds with Trp275, Tyr82 and Trp260 of ATX (arrows).

To further rationalize the SAR data obtained and confirm our proposed binding mode hypothesis, we determined the crystal structure of ATX bound to **5** and **11** to 2.4 Å and 2.1 Å resolution, respectively (Figure 3 A-D and Figure 4 A and B). These structures fully support our original design hypothesis and demonstrate that the hydrophobic anchor, the 3,5-dichlorobenzyl group, occupies essentially the same position in the hydrophobic pocket in the structures of **3**, **5**, and **11**. Notably, the steroid moiety also remains in a similar, albeit not identical binding orientation between structures, when comparing the structures of **1**, **5**, and **11**, and is held in position by several interactions including two hydrogen bonds, with the

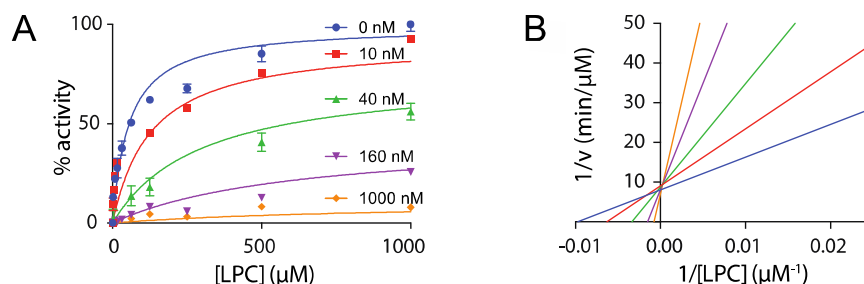
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3 Tyr82 and Trp260 side chains. The piperazine linker unit, however, adopts a significantly  
4 different conformation in **11** compared to **3**, projecting towards the tunnel, instead of the  
5 catalytic site; the linear linker in **5** follows a similar vector to that of the piperazine in **11**.  
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7 Interestingly, the linker region in both structures does not exhibit well defined electron  
8 density compared to the other groups, suggesting a certain degree of structural flexibility in  
9 the region, with the linker potentially adopting several conformations. Accordingly, these  
10 structures suggest that the bile salt steroid moiety and the dichlorobenzyl group act as  
11 anchors in the tunnel and the pocket, respectively, and optimization of binding should  
12 subsequently focus on the linker region to provide a conformation which may lead to optimal  
13 binding. Based on this, the 3,5-dichlorobenzyl group was retained in subsequent analogues  
14 prepared, and our focus turned to tuning the spacer region of our nascent series.

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17 Several isosteric cores were evaluated (**12-20**, Table 1) with a range of ATX inhibitory  
18 activity noted. Aminomethyl piperidine systems were tolerated with a preference for the  
19 exocyclic carbamate (*cf.* **12-13**), and more constrained systems such as **14** led to a 10-fold  
20 reduction in activity. Other isosteres such as azetidiny and 3-amino pyrrolidine (**15-20**)  
21 showed comparable levels of potency relative to the progenitor compound **4**. A notable  
22 exception is evidenced by compound **17**, displaying an  $IC_{50}$  of 20 nM against ATX mediated  
23 production of LPA, which compares favorably to **2** ( $IC_{50} = 3 \text{ nM}^{10}$ ). Additionally, This  
24 represents an over 500-fold improvement in activity compared to the parent bile acid, and  
25 demonstrates the utility of a structure based design approach in expediently optimizing a  
26 weak endogenous modulator of ATX activity towards a highly potent lead candidate.

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29 The crystal structure of **17** bound to ATX (Figure 3 E and F, Figure 4 C) was determined to  
30 1.95 Å resolution, and illustrates that similar to the structures of **5** and **11**, the 3,5-  
31 dichlorobenzyl and the steroid groups again act as anchors in the pocket and the tunnel,  
32 respectively, making several contacts with ATX, and occupying essentially the same  
33 positions as in complexes of **5** and **11**. The two hydrogen bonds between ATX and the  
34 steroid moiety of hybrid inhibitors remain the same in the complex of ATX with **17** (Figure 4  
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C) as for the complexes with **5** and **11**; however, the positioning of the new linker allows the formation of an additional hydrogen bond between the urethane carbonyl and the main chain amide of Trp275. This observation could account in part for the increased potency of **17**. In addition, the electron density for the linker region is better defined in this structure, indicating a more restricted spatial distribution of the linker. The linker of **17** also has a better fit to the ATX surface (compare Figure 3 E to 3 A, C). Collectively, these structural observations account for the superior activity of **17**.

To further characterize the potency and mechanism of inhibition, we examined inhibition of LPC hydrolysis by **17** in greater depth, based on measurement of a single LPA species, (18:1, referring to an 18 carbon chain with a single degree of unsaturation). As anticipated, **17** was shown to be a competitive inhibitor of LPC hydrolysis (Figure 5) with a  $K_i$  of 0.006  $\mu\text{M}$ . This contrasts with the progenitor steroid **1** which is a weak non-competitive inhibitor of LPC hydrolysis. Accordingly, by targeting the hydrophobic pocket, we have switched the mechanism of action in this emerging lead series compared to the endogenous modulator **1**.

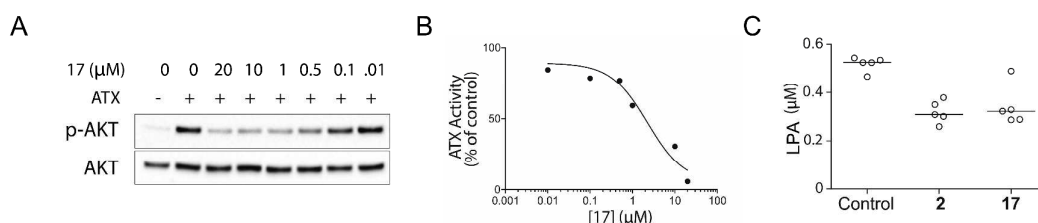


**Figure 5.** **17** acts as a competitive inhibitor of LPC hydrolysis. (A) ATX lysoPLD activity with no inhibitor (blue symbols) and with four concentrations of **17** as a function of LPC (18:1) substrate concentrations; the lines represent the non-linear regression fit of all data (B) a simulated Lineweaver-Burk plot, fitting the velocity for each experiment separately, graphically demonstrating that **17** acts as a competitive inhibitor of LPC hydrolysis with a  $K_i$  of  $6 \pm 2$  nM.

Having developed a novel tool compound with known mechanism of action, we subsequently sought to demonstrate that **17** inhibits LPA-mediated signaling in cancer cells, by examining its ability to block phosphorylation of the AKT kinase; consistently, concentrations of above 0.1  $\mu\text{M}$  of **17** decreases the level of pAKT, as detected by Western

blot analysis (Figure 6 A), suggesting that compound **17** is a viable tool in a physiologically relevant context.

Lastly, in order to examine inhibition of ATX *in vivo*, **17**, and **2** as a positive control, were administered to mice (10 mg/kg, i.p.) and the levels of eight abundant LPA species were summed and determined by mass spectrometry.<sup>15</sup> The *in vivo* activity of **17** was similar to that of **2**, reducing LPA levels to about half to that of control mice injected with vehicle alone (Figure 6 B). Although determination of *in vivo* half-life was not possible in this experiment, the data indicates that compound **17** must have sufficient degree of stability in order to be able to modulate LPA levels 8 hours following i.p. administration. This highly encouraging data indicates robust target engagement in an *in vivo* setting, thus validating our emerging lead series as promising tools to further prosecute the biology of Autotaxin in a variety of disease states.



**Figure 6.** (A) Western-blot showing inhibition of ATX by **17** decreases Ser473 phosphorylation of AKT in BJEH cells. (B) Graphical depiction of Western-blot using densitometry. (C) LPA levels measured for the injection of control, **2** and **17**, showing the levels of LPA in the individual animals (circles) and the median value (horizontal line); Student's unpaired two tailed T-test suggests the difference of **2** and **17** to the control is significant ( $p < 0.01$ ).

In summary, we report the development of an endogenous steroid modulator of Autotaxin function towards a highly potent hybrid lead series with significant levels of activity *in vivo*. The optimization trajectory was significantly expedited through application of a structure-based design approach, leading to the rapid identification of compound **17** which could be differentiated from the progenitor steroid both in terms of significantly enhanced potency and mechanism of action (competitive vs non-competitive inhibition of LPA hydrolysis). The

structural data generated confirmed the original design hypothesis, and support the observed mechanism of action for the series lead, compound **17**. Inhibition of ATX activity by our lead compound in cells and in a pilot experiment in mice, suggest that these new entities will serve as valuable tools in further understanding the pathophysiological roles of Autotaxin.

## Experimental

**Chemistry. General.** All reagents and solvents were obtained from commercial suppliers and were used without further purification unless otherwise stated. Purification was carried out according to standard laboratory methods. Reactions were carried out using conventional glassware room temperature was generally 18 °C. Reactions were carried out at elevated temperatures using a temperature-regulated hotplate/stirrer. Thin layer chromatography was carried out using Merck silica plates coated with fluorescent indicator UV254. These were analyzed under 254 nm UV light or developed using potassium permanganate solution. Normal phase flash chromatography was carried out using ZEOprep 60 HYD 40-63  $\mu\text{m}$  silica gel. Fourier Transformed Infra-Red (FTIR) spectra were obtained on a Shimadzu IRAffinity-1 machine.  $^1\text{H}$  and  $^{13}\text{C}$ , NMR spectra were obtained on a Bruker AV 400 at 400 MHz and 101 MHz, respectively and a Bruker AVIIIHD500 at 500 MHz and 126 MHz, respectively. Chemical shifts are reported in ppm and coupling constants are reported in Hz with  $\text{CDCl}_3$  referenced at 7.26 ( $^1\text{H}$ ) and 77.1 ppm ( $^{13}\text{C}$ ) and  $\text{DMSO}-d_6$  referenced at 2.50 ( $^1\text{H}$ ) and 39.52 ppm ( $^{13}\text{C}$ ). Compound purity was determined by LCMS and NMR analysis, and all compounds were  $\geq 95\%$  purity. High-resolution mass spectra were obtained through analysis at the EPSRC UK National Mass Spectrometry Facility at Swansea University or in the School of Chemistry at the University of Glasgow, UK.

**General Procedure A: Carbamate Formation.** For example, for the preparation of 1-(*tert*-butyl) 4-(3,5-dichlorobenzyl) piperazine-1,4-dicarboxylate, **21**.<sup>11</sup> To a round bottom flask was added (3,5-dichlorophenyl)methanol (1.04 g, 5.91 mmol, 1.10 equiv), 1,1'-carbonyldiimidazole (0.96 g, 5.91 mmol, 1.10 equiv) in DMF (5 mL) and stirred for 30 min at room temperature. To the reaction mixture was added *tert*-butyl piperazine-1-carboxylate (1.00 g, 5.37 mmol, 1.00 equiv) and stirred for 30 min at room temperature. The reaction was diluted with  $\text{H}_2\text{O}$  (10 mL) and the precipitate filtered under vacuum with  $\text{H}_2\text{O}$ , and dried in a dessicator to afford the desired product as a white solid (1.87 g, 90 %).  $\nu_{\text{max}}$  (neat):

2847, 1698, 1678, 1418  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.34 (t,  $J$  = 1.9 Hz, 1H), 7.25 (d,  $J$  = 1.91 Hz, 2H), 5.10 (s, 2H), 3.55-3.48 (m, 4H), 3.48-3.42 (m, 4H), 1.49 (s, 9H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  154.8, 154.7, 140.0, 153.3, 128.4, 126.3, 80.4, 65.8, 43.9, 28.5.  $1 \times \text{C}$  not observed. HRMS: exact mass calculated for  $[\text{M}+\text{NH}_4]^+$  ( $\text{C}_{17}\text{H}_{26}\text{Cl}_2\text{N}_3\text{O}_4$ ) requires  $m/z$  406.1295, found  $m/z$  406.1293.

**General Procedure B: *N*-Benzyl Carbamate Formation.** For example, for the preparation of benzyl *tert*-butyl ethane-1,2-diylldicarbamate, **22**.<sup>11</sup> To a round bottom flask was added *tert*-butyl 3-methylpiperazine-1-carboxylate (100 mg, 0.50 mmol, 1.00 equiv), *N*-(benzyloxycarbonyloxy)succinimide (137 mg, 0.55 mmol, 1.10 equiv) and  $\text{NaHCO}_3$  (66.4 mg, 0.75 mmol, 1.40 equiv), in THF: $\text{H}_2\text{O}$  (1 mL:1 mL) and stirred for 16 h at room temperature. The reaction mixture was diluted with  $\text{H}_2\text{O}$  (20 mL) and the organics were extracted with ethyl acetate (2  $\times$  20 mL), dried with a hydrophobic frit and concentrated *in vacuo* to afford the desired product as a clear oil (154 mg, 89%).  $\nu_{\text{max}}$  (neat): 3314, 2977, 2931, 1689, 1546  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.39-7.28 (m, 5H), 5.29 (s, 1H), 5.09 (s, 2H), 4.94 (s, 1H), 3.32-3.15 (m, 4H), 1.42 (s, 9H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  156.3, 155.9, 136.0, 128.0, 127.63, 127.60, 79.1, 66.2, 41.0, 40.1, 27.9. HRMS: exact mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{15}\text{H}_{23}\text{N}_2\text{O}_4$ ) requires 295.1652  $m/z$ , found 295.1654  $m/z$ .

**General Procedure C: *N*-Boc deprotection.** For example, for the preparation of 3,5-dichlorobenzyl piperazine-1-carboxylate, **23**.<sup>11</sup> To a round bottom flask was added 1-(*tert*-butyl) 4-(3,5-dichlorobenzyl)piperazine-1,4-dicarboxylate (1.87g, 4.80 mmol), a mixture of trifluoroacetic acid: $\text{CH}_2\text{Cl}_2$  (1 mL:1mL) and the reaction stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* and purified by strong cation exchange eluting with 2 M  $\text{NH}_3$  in MeOH to afford the desired product as a white solid (0.53 g, 60%). Consistent with previously reported spectral analysis.  $\nu_{\text{max}}$  (neat): 2918, 2849, 2494, 1670, 1425, 1128  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.26 (t,  $J$  = 1.7 Hz, 1H), 7.20 (d,  $J$  = 1.8 Hz,



2H), 5.04 (s, 2H), 3.51-3.45 (m, 4H), 2.88-2.81 (m, 4H), 2.02 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  154.7, 140.1, 135.1, 128.2, 126.1, 65.6, 45.5, 44.4. HRMS: exact mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{12}\text{H}_{15}\text{Cl}_2\text{N}_2\text{O}_2$ ) requires  $m/z$  289.0505, found  $m/z$  289.0503.

**General Procedure D: HATU amidation.** For example, for the preparation of, 3,5-dichlorobenzyl-4-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)piperazine-1-carboxylate, **4**. To a round bottom flask was added ursodeoxycholic acid (100 mg, 0.26 mmol, 1.1 equiv) in DMF (1 mL), *N,N*-diisopropylethylamine (180  $\mu\text{L}$ , 1.02 mmol, 4.0 equiv) and then 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 97 mg, 0.26 mmol, 1.1 equiv). After stirring at room temperature for 15 min 3,5-dichlorobenzyl piperazine-1-carboxylate (67 mg, 0.23 mmol, 1.0 equiv) was added and stirred for 16 h at room temperature. The reaction mixture was diluted with  $\text{H}_2\text{O}$  (20 mL) and the resulting precipitate filtered under vacuum. The crude material was purified by column chromatography on silica (0-10% MeOH in ethyl acetate) to afford the desired product as a clear solid (87 mg, 51%).  $\nu_{\text{max}}$  (neat): 3496, 2931, 2866, 1708, 1637, 1421, 1231  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz):  $\delta$  7.32-7.30 (m, 1H), 7.24-7.21 (m, 2H), 5.08 (s, 2H), 3.65-3.44 (m, 9H), 2.42-2.32 (m, 1H), 2.28-2.18 (m, 1H), 2.02-1.96 (m, 1H), 1.96-1.85 (m, 1H), 1.85-1.74 (m, 4H), 1.74-1.63 (m, 3H), 1.63-1.55 (m, 2H), 1.53-1.39 (m, 6H), 1.38-1.22 (m, 5H), 1.17-0.98 (m, 3H), 0.96-0.91 (m, 6H), 0.67 (s, 3H), 2  $\times$  H not observed (exchangeable).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  172.4, 154.8, 139.9, 135.3, 128.5, 126.4, 71.54, 71.47, 66.0, 55.9, 55.1, 45.4, 43.9, 42.6, 40.3, 39.3, 37.4, 37.0, 35.6, 35.1, 34.2, 31.5, 30.5, 28.8, 27.0, 23.5, 21.3, 18.8, 12.3. 2  $\times$  C not observed. HRMS: exact mass calculated for  $[\text{M}+\text{Na}]^+$  ( $\text{C}_{36}\text{H}_{52}\text{Cl}_2\text{N}_2\text{NaO}_5$ ) requires 685.3145  $m/z$ , found 685.3119  $m/z$ .

**3,5-Dichlorobenzyl (2-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-**

**yl)pentanamido)ethyl)carbamate (5).** Prepared according to General Procedure **D** with ursodeoxycholic acid (176 mg, 0.45 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (286  $\mu$ L, 1.64 mmol, 4.0 equiv), HATU (171 mg, 0.45 mmol, 1.1 equiv), 3,5-dichlorobenzyl (2-aminoethyl)carbamate (107 mg, 0.41 mmol, 1.0 equiv) and DMF (2 mL). The crude material was subjected to the purification procedure outlined in the General Procedure (silica gel, 0-10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to give the desired product as a clear solid (134 mg, 51 %).  $\nu_{\text{max}}$  (neat): 3329, 2933, 2864, 1706, 1652, 1574  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.30-7.27 (m, 1H), 7.23-7.19 (m, 2H), 6.34 (br. s, 1H), 5.73 (br. s, 1H), 5.01 (s, 2H), 3.65-3.50 (m, 2H), 3.42-3.25 (m, 4H), 2.27-2.12 (m, 1H), 2.11-2.00 (m, 1H), 2.00-1.93 (m, 1H), 1.88-1.71 (m, 5H), 1.70-1.61 (m, 2H), 1.60-1.53 (m, 2H), 1.50-1.35 (m, 5H), 1.35-1.16 (m, 6H), 1.14-0.98 (m, 3H), 0.97-0.87 (m, 6H), 0.64 (s, 3H), 2  $\times$  H not observed (exchangeable).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  175.0, 156.9, 140.1, 135.2, 128.2, 126.1, 71.4, 65.1, 56.01, 55.95, 55.1, 43.8, 42.6, 41.2, 40.3, 39.9, 39.4, 37.5, 37.2, 35.6, 35.1, 34.2, 33.6, 32.0, 30.4, 28.8, 27.0, 23.5, 21.3, 18.6, 12.2. 1  $\times$  C not observed. HRMS: exact mass calculated for  $[\text{M}+\text{Na}]^+$  ( $\text{C}_{34}\text{H}_{50}\text{Cl}_2\text{N}_2\text{NaO}_5$ ) requires 659.2989  $m/z$ , found 659.2965  $m/z$ .

**3,5-Dichlorobenzyl (3-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-**

**yl)pentanamido)propyl)carbamate (6).** Prepared according to General Procedure **D** with ursodeoxycholic acid (156 mg, 0.39 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (314  $\mu$ L, 1.8 mmol, 4.0 equiv), HATU (151 mg, 0.39 mmol, 1.1 equiv), 3,5-dichlorobenzyl (3-aminopropyl)carbamate (100 mg, 0.36 mmol, 1.0 equiv) and DMF (1 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to give the desired product as a clear solid (39 mg, 16 %).  $\nu_{\text{max}}$  (neat): 3813, 2933, 2866, 1708, 1654, 1574, 1542  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.31-7.28 (m, 1H), 7.25-7.21 (m, 2H), 5.92 (s, 1H), 5.33 (s, 1H), 5.03 (s, 2H), 3.62-3.55 (m, 2H), 3.51-3.46 (m, 2H), 3.35-3.31 (m, 2H), 3.26-3.18 (m, 2H), 2.29-2.20 (m, 1H), 2.16-2.04 (m,

1H), 2.03-1.96 (m, 1H), 1.95-1.86 (m, 1H), 1.84-1.74 (m, 4H), 1.71-1.55 (m, 7H), 1.53-1.39 (m, 6H), 1.37-1.21 (m, 4H), 1.14 (td,  $J = 3.1, 12.6$  Hz, 1H), 1.10-0.98 (m, 2H), 0.97-0.90 (m, 6H), 0.67 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  174.4, 156.6, 140.3, 135.2, 128.2, 126.1, 71.6, 71.5, 65.1, 55.9, 55.1, 43.9, 42.6, 40.3, 39.3, 37.7, 37.5, 37.0, 36.0, 35.6, 35.1, 34.2, 33.8, 32.0, 30.5, 30.2, 28.8, 27.0, 23.5, 21.3, 18.6, 12.3.  $1 \times \text{C}$  not observed. HRMS: exact mass calculated for  $[\text{M}+\text{Na}]^+$  ( $\text{C}_{35}\text{H}_{52}\text{Cl}_2\text{N}_2\text{NaO}_5$ ) requires 673.3145  $m/z$ , found 673.3113  $m/z$ .

**(4*R*)-*N*-(3,5-Dichlorobenzyl)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamide (7).**

Prepared according to General Procedure **D** with ursodeoxycholic acid (100 mg, 0.26 mmol, 1.0 equiv), *N,N*-diisopropylethylamine (180  $\mu\text{L}$ , 1.02 mmol, 4.0 equiv), HATU (97 mg, 0.26 mmol, 1.0 equiv), 3,5-dichlorobenzylamine (35  $\mu\text{L}$ , 0.26 mmol, 1.0 equiv) and DMF (1 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to afford the desired product as a white solid (14 mg, 10%).  $\nu_{\text{max}}$  (neat): 3296, 2931, 2866, 1654, 1574, 1434  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.28-7.25 (m, 1H), 7.15 (d,  $J = 1.9$  Hz, 2H), 5.80 (s, 1H), 4.39 (d,  $J = 6.1$  Hz, 2H), 3.64-3.54 (m, 2H), 2.35-2.25 (m, 1H), 2.18-2.09 (m, 1H), 1.99 (dt,  $J = 3.0, 12.4$  Hz, 1H), 1.94-1.85 (m, 1H), 1.85-1.74 (m, 3H), 1.70-1.63 (m, 2H), 1.62-1.55 (m, 3H), 1.53-1.39 (m, 6H), 1.38-1.20 (m, 6H), 1.17-0.97 (m, 3H), 0.96-0.91 (m, 6H), 0.67 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  173.6, 142.1, 135.4, 127.8, 126.2, 71.6, 71.5, 55.9, 55.1, 43.9, 42.7, 42.6, 40.3, 39.3, 37.5, 37.0, 35.5, 35.1, 34.2, 33.7, 31.9, 30.5, 28.8, 27.0, 23.5, 21.3, 18.6, 12.3.  $1 \times \text{C}$  not observed. HRMS: exact mass calculated for  $[\text{M}+\text{Na}]^+$  ( $\text{C}_{31}\text{H}_{45}\text{Cl}_2\text{NNaO}_3$ ) requires 572.2669  $m/z$ , found 572.2650  $m/z$ .

**(4*R*)-*N*-(2-Aminoethyl)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamide (8).**

Prepared according to General Procedure **C**, using *tert*-butyl (2-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-

3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamido)ethyl)carbamate (936 mg, 1.75 mmol, 1.0 equiv), trifluoroacetic acid (2 mL), CH<sub>2</sub>Cl<sub>2</sub> (4 mL), diluted with 1 M NaOH (100 mL), the organics were extracted with ethyl acetate (100 mL), washed with brine (100 mL), dried with a hydrophobic frit and concentrated *in vacuo* to afford the desired product as a white solid (605 mg, 63 %).  $\nu_{\max}$  (neat): 3283, 2935, 2669, 1715, 1654, 1157 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz):  $\delta$  9.36 (s, 1H), 7.89 (s, 1H), 4.42 (d, *J* = 4.6 Hz, 1H), 3.85 (d, *J* = 6.8 Hz, 1H), 3.21 (t, *J* = 5.4 Hz, 2H), 3.17 (t, *J* = 5.5 Hz, 2H), 2.13-2.03 (m, 1H), 1.99-1.90 (m, 2H), 1.86-1.80 (m, 1H), 1.86-1.80 (m, 1H), 1.80-1.71 (m, 1H), 1.70-1.60 (m, 3H), 1.51-1.43 (m, 3H), 1.42-1.26 (m, 7H), 1.23-1.06 (m, 3H), 1.03-0.92 (m, 2H), 0.89-0.85 (m, 6H), 0.61 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz):  $\delta$  173.0, 69.7, 69.4, 55.9, 54.7, 43.1, 43.0, 42.2, 39.8, 39.2, 38.7, 37.7, 37.30, 37.26, 34.9, 34.8, 33.7, 32.4, 31.5, 30.2, 28.1, 26.7, 23.3, 20.8, 18.4, 12.0. HRMS: exact mass calculated for [M+H]<sup>+</sup> (C<sub>26</sub>H<sub>47</sub>N<sub>2</sub>O<sub>3</sub>) requires 435.3581 *m/z*, found 435.3576 *m/z*.

**Benzyl (2-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamido)ethyl)carbamate (9).** Prepared according to General Procedure **D** with ursodeoxycholic acid (445 mg, 1.13 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (720  $\mu$ L, 4.12 mmol, 4.0 equiv), HATU (430 mg, 0.13 mmol, 1.1 equiv), benzyl (2-aminoethyl)carbamate (200 mg, 1.03 mmol, 1.0 equiv) and DMF (5 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford the desired product as a clear solid (399 mg, 68 %).  $\nu_{\max}$  (neat): 3298, 2933, 2866, 1706, 1652, 1527 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz):  $\delta$  7.39-7.28 (m, 5H), 6.10 (s, 1H), 5.29 (s, 1H), 5.09 (s, 2H), 3.63-3.52 (m, 2H), 3.41-3.28 (m, 4H), 2.21-2.16 (m, 1H), 2.08-2.01 (m, 1H), 2.01-1.96 (m, 1H), 1.91-1.84 (m, 1H), 1.82-1.75 (m, 3H), 1.73-1.64 (m, 4H), 1.61-1.54 (m, 3H), 1.52-1.39 (m, 5H), 1.34-1.20 (m, 6H), 1.16-1.09 (m, 1H), 1.06-0.99 (m, 2H), 0.95-0.88 (m, 6H), 0.67 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>):  $\delta$  174.6, 157.4, 136.5, 128.7, 128.3, 128.2, 71.6, 71.5, 67.0, 55.9, 55.1, 43.9, 42.6, 41.2, 40.3, 39.4,

37.5, 37.0, 35.6, 35.1, 34.2, 33.7, 31.9, 30.5, 28.8, 27.0, 23.5, 21.3, 18.6, 12.3. 2 × C not observed. HRMS: exact mass calculated for  $[M+Na]^+$  ( $C_{34}H_{52}N_2NaO_5$ ) requires 591.3768  $m/z$ , found 591.3746  $m/z$ .

**Benzyl-4-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)piperazine-1-carboxylate (10).** Prepared according to General Procedure **D** with ursodeoxycholic acid (500 mg, 1.27 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (450  $\mu$ L, 2.57 mmol, 2.0 equiv), HATU (243 mg, 1.27 mmol, 1.1 equiv), benzylpiperazine-1-carboxylate (223  $\mu$ L, 1.16 mmol, 1.0 equiv) and DMF (5 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $CH_2Cl_2$ ) to afford the desired product as a clear solid (258 mg, 37%).  $\nu_{max}$  (neat): 3374, 2929, 2866, 1702, 1630  $cm^{-1}$ .  $^1H$  NMR ( $CDCl_3$ , 500 MHz):  $\delta$  7.39-7.30 (m, 5H), 5.15 (s, 2H), 3.63-3.54 (m, 4H), 3.56-3.40 (m, 7H), 2.41-2.33 (m, 1H), 2.26-2.17 (m, 1H), 1.99 (d,  $J$  = 12.5 Hz, 1H), 1.95-1.85 (m, 1H), 1.84-1.73 (m, 4H), 1.71-1.63 (m, 3H), 1.62-1.55 (m, 2H), 1.52-1.39 (m, 6H), 1.39-1.21 (m, 5H), 1.19-0.97 (m, 3H), 0.97-0.91 (m, 6H), 0.67 (s, 3H).  $^{13}C$  NMR ( $CDCl_3$ , 101 MHz):  $\delta$  172.4, 155.3, 136.5, 128.7, 128.4, 128.2, 71.6, 71.5, 67.6, 55.9, 55.1, 45.5, 43.9, 42.6, 41.4, 40.3, 39.3, 37.4, 37.0, 35.6, 35.1, 34.2, 31.5, 30.5, 28.8, 27.0, 23.5, 21.3, 18.8, 12.3. 2 × aliphatic C not observed. HRMS: exact mass calculated for  $[M+Na]^+$  ( $C_{36}H_{54}N_2NaO_5$ ) requires 617.3925  $m/z$ , found 617.3915  $m/z$ .

**3,5-Dichlorobenzyl-4-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentyl)piperazine-1-carboxylate (11).** To a round bottom flask at 0 °C was added 3,5-dichlorobenzyl 4-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)piperazine-1-carboxylate (92 mg, 0.14 mmol, 1.0 equiv), THF (5 mL),  $BH_3$ .THF (1 M, 700  $\mu$ L, 0.70 mmol, 5.0 equiv) and the reaction stirred at 70 °C for 16 h. The reaction mixture was cooled to room temperature, quenched with 1 M

HCl (20 mL) and stirred for 1 h. The organics were extracted with ethyl acetate (20 mL), washed with brine (20 mL), dried with a hydrophobic frit and concentrated *in vacuo*. The crude material was purified by column chromatography on silica (0-100% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford the desired product as a white solid (4 mg, 4%).  $\nu_{\max}$  (neat): 3381, 2929, 2866, 1708, 1635 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz):  $\delta$  7.33-7.31 (m, 1H), 7.23 (d, *J* = 1.7 Hz, 2H), 5.08 (s, 2H), 3.66-3.43 (m, 12H), 2.43-2.34 (m, 1H), 2.29-2.18 (m, 1H), 2.03-1.97 (m, 1H), 1.96-1.86 (m, 1H), 1.85-1.74 (m, 4H), 1.71-1.62 (m, 2H), 1.62-1.53 (m, 4H), 1.52-1.41 (m, 6H), 1.39-1.32 (m, 2H), 1.29-1.21 (m, 2H), 1.19-1.11 (m, 2H), 1.11-1.01 (m, 2H), 0.99-0.92 (m, 6H), 0.68 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 126 MHz):  $\delta$  172.4, 139.9, 135.3, 128.5, 126.4, 71.6, 71.5, 66.0, 55.9, 55.1, 51.0, 45.5, 43.9, 42.6, 40.3, 39.3, 37.5, 37.0, 35.7, 35.1, 34.2, 31.5, 30.5, 28.9, 27.1, 23.5, 21.3, 18.8, 12.3. 3 × C not observed. HRMS: exact mass calculated for [M+H]<sup>+</sup> (C<sub>36</sub>H<sub>55</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>4</sub>) requires 649.3533 *m/z*, found 649.3506 *m/z*.

**3,5-Dichlorobenzyl-4-(((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-**

**yl)pentanamido)methyl)piperidine-1-carboxylate (12).** Prepared according to General Procedure **D** with ursodeoxycholic acid (136 mg, 0.35 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (223  $\mu$ L, 1.28 mmol, 4.0 equiv), HATU (133 mg, 0.35 mmol, 1.1 equiv), 3,5-dichlorobenzyl 4-(aminomethyl)piperidine-1-carboxylate (100 mg, 0.32 mmol, 1.0 equiv) and DMF (2 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford the desired product as a clear solid (80 mg, 36 %).  $\nu_{\max}$  (neat): 3323, 2931, 2866, 1691, 1654 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  7.31-7.29 (m, 1H), 7.23-7.20 (m, 2H), 5.52 (s, 1H), 5.05 (s, 2H), 4.27-4.09 (m, 2H), 3.65-3.53 (m, 2H), 3.26-3.08 (m, 2H), 2.89-2.79 (m, 2H), 2.30-2.21 (m, 1H), 2.14-2.03 (m, 1H), 2.02-1.96 (m, 1H), 1.95-1.86 (m, 1H), 1.85-1.75 (m, 3H), 1.74-1.63 (m, 4H), 1.63-1.55 (m, 4H), 1.55-1.39 (m, 6H), 1.38-1.12 (m, 7H), 1.10-0.98 (m, 2H), 0.97-0.90 (m, 6H), 0.67 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz):  $\delta$  173.8, 154.8, 140.4, 135.2, 128.2, 126.2, 71.6,

71.5, 65.6, 55.9, 55.1, 44.9, 44.0, 43.9, 42.6, 40.3, 39.3, 37.5, 37.0, 36.3, 35.5, 35.1, 34.2, 33.8, 32.0, 30.5, 29.8, 28.8, 27.0, 23.5, 21.3, 28.6, 12.3.  $1 \times C$  not observed. HRMS: exact mass calculated for  $[M+Na]^+$  ( $C_{38}H_{56}Cl_2N_2NaO_5$ ) requires 713.3458  $m/z$ , found 713.3425  $m/z$ .

**3,5-Dichlorobenzyl ((1-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)-4-**

**hydroxypiperidin-4-yl)methyl)carbamate (13).** Prepared according to General Procedure **D** with ursodeoxycholic acid (67 mg, 0.17 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (131  $\mu$ L, 0.75 mmol, 5.0 equiv), HATU (65 mg, 0.17 mmol, 1.1 equiv), 3,5-dichlorobenzyl ((4-hydroxypiperidin-4-yl)methyl)carbamate (50 mg, 0.15 mmol, 1.0 equiv) and DMF (1 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $CH_2Cl_2$ ) to afford the desired product as a clear solid (5.1 mg, 3 %).  $\nu_{max}$  (neat): 3357, 2929, 2866, 1702, 1618, 1574  $cm^{-1}$ .  $^1H$  NMR ( $CDCl_3$ , 400 MHz):  $\delta$  7.34-7.28 (m, 1H), 7.25 (d,  $J = 1.7$  Hz, 2H), 5.28 (s, 1H), 5.05 (s, 2H), 4.26 (d,  $J = 12.9$  Hz, 1H), 3.64-3.54 (m, 3H), 3.43 (t,  $J = 11.4$  Hz, 1H), 3.34-3.25 (m, 1H), 3.22-3.15 (m, 1H), 3.11 (t,  $J = 11.4$  Hz, 1H), 2.45-2.34 (m, 1H), 2.32-2.19 (m, 1H), 2.04-1.97 (m, 1H), 1.96-1.86 (m, 1H), 1.85-1.73 (m, 4H), 1.69-1.64 (m, 2H), 1.62-1.54 (m, 6H), 1.53-1.47 (m, 3H), 1.46-1.40 (m, 4H), 1.38-1.28 (m, 5H), 1.19-0.98 (m, 3H), 0.97-0.90 (m, 6H), 0.70 (s, 3H).  $^{13}C$  NMR ( $CDCl_3$ , 101 MHz):  $\delta$  172.2, 157.3, 139.8, 135.3, 128.4, 126.2, 71.6, 71.5, 70.4, 65.6, 55.9, 55.1, 51.4, 43.9, 42.6, 41.7, 40.3, 39.3, 37.5, 37.4, 37.0, 35.7, 35.5, 35.1, 34.8, 34.2, 31.7, 30.52, 30.48, 28.8, 27.1, 23.5, 21.3, 18.8, 12.3. Rotameric mixture observed. HRMS: exact mass calculated for  $[M+Na]^+$  ( $C_{38}H_{56}Cl_2N_2NaO_6$ ) requires 729.3408  $m/z$ , found 729.3372  $m/z$ .

**2-(3,5-Dichlorobenzyl)-7-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-**

**yl)pentanoyl)hexahydroimidazo[1,5-*a*]pyrazin-3(2*H*)-one (14).** Prepared according to General Procedure **D** with ursodeoxycholic acid (120 mg, 0.31 mmol, 1.1 equiv), *N,N*-

diisopropylethylamine (244  $\mu$ L, 1.50 mmol, 5.0 equiv), HATU (117 mg, 0.31 mmol, 1.1 equiv), 2-(3,5-dichlorobenzyl)hexahydroimidazo[1,5-*a*]pyrazin-3(2*H*)-one (73 mg, 0.32 mmol, 1.0 equiv) and DMF (2 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to afford the desired product as a clear solid as an epimeric mixture (80 mg, 36%).  $\nu_{\text{max}}$  (neat): 3404, 2931, 2864, 1691, 1635, 1429  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz, 323 K):  $\delta$  7.30-7.27 (m, 1H), 7.16-7.12 (m, 2H), 4.72-4.57 (m, 1H), 4.38 (d,  $J$  = 15.4 Hz, 1H), 4.29 (d,  $J$  = 15.5 Hz, 1H), 3.95 (d,  $J$  = 12.1 Hz, 1H), 3.84-3.71 (m, 1H), 3.64-3.53 (m, 3H), 3.35 (t,  $J$  = 8.6 Hz, 1H), 3.20-3.04 (m, 1H), 3.01-2.84 (m, 2H), 2.43-2.32 (m, 1H), 2.32-2.19 (m, 1H), 2.00 (d,  $J$  = 12.5 Hz, 1H), 1.95-1.75 (m, 5H), 1.71-1.65 (m, 2H), 1.63-1.56 (m, 2H), 1.56-1.40 (m, 8H), 1.38-1.02 (m, 8H), 1.00-0.92 (m, 6H), 0.67 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  172.6, 159.0, 140.5, 135.5, 128.0, 126.5, 71.51, 71.46, 60.5, 55.8, 55.1, 50.8, 47.3, 46.0, 45.5, 45.3, 43.9, 42.5, 41.2, 40.3, 39.3, 37.4, 37.0, 35.6, 35.0, 34.2, 31.5, 30.5, 30.4, 28.8, 27.0, 23.5, 21.3, 18.8, 12.3. HRMS: exact mass calculated for  $[\text{M}+\text{Na}]^+$  ( $\text{C}_{37}\text{H}_{53}\text{Cl}_2\text{N}_3\text{NaO}_4$ ) requires 696.3305  $m/z$ , found 696.3289  $m/z$ .

**3,5-Dichlorobenzyl-3-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamido)azetidine-1-carboxylate (15).** Prepared according to General Procedure **D** with ursodeoxycholic acid (157 mg, 0.40 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (314  $\mu$ L, 1.8 mmol, 4.0 equiv), HATU (152 mg, 0.40 mmol, 1.1 equiv), 3,5-dichlorobenzyl 3-aminoazetidine-1-carboxylate (100 mg, 0.36 mmol, 1.0 equiv) and DMF (1 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to afford the desired product as a clear solid (108 mg, 46 %).  $\nu_{\text{max}}$  (neat): 3400, 2933, 2865, 1699, 1654, 1574, 1419  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz):  $\delta$  7.30-7.27 (m, 1H), 7.21-7.17 (m, 2H), 6.51 (br. s, 1H), 5.00 (s, 2H), 4.69-4.62 (m, 1H), 4.35-4.28 (m, 2H), 3.83 (dd,  $J$  = 5.2, 9.3 Hz, 2H), 3.60-3.51 (m, 2H), 2.31-2.19 (m, 1H), 2.12-2.02 (m, 1H), 2.02-1.94 (m, 1H), 1.93-1.84 (m, 3H), 1.82-1.72 (m, 4H), 1.69-1.62 (m, 2H), 1.61-1.54 (m, 2H), 1.50-1.45 (m, 2H), 1.44-



1.39 (m, 4H), 1.35-1.18 (m, 5H), 1.16-0.98 (m, 3H), 0.95-0.88 (m, 6H), 0.65 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  173.8, 155.7, 140.0, 135.2, 128.3, 126.2, 71.43, 71.38, 65.2, 56.0, 55.1, 50.8, 43.9, 43.8, 42.6, 40.3, 39.9, 39.4, 37.5, 37.1, 35.6, 35.0, 34.2, 33.4, 31.8, 30.4, 28.8, 27.0, 23.5, 21.3, 18.6, 12.3. HRMS: exact mass calculated for  $[\text{M}+\text{Na}]^+$  ( $\text{C}_{35}\text{H}_{50}\text{Cl}_2\text{N}_2\text{NaO}_5$ ) requires 671.2989  $m/z$ , found 671.2957  $m/z$ .

**3,5-Dichlorobenzyl** (1-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)azetidin-3-yl)carbamate (**16**). Prepared according to General Procedure **D** with ursodeoxycholic acid (157 mg, 0.40 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (314  $\mu\text{L}$ , 1.8 mmol, 4.0 equiv), HATU (152 mg, 0.40 mmol, 1.1 equiv), 3,5-dichlorobenzyl azetidin-3-ylcarbamate (100 mg, 0.36 mmol, 1.0 equiv) and DMF (1 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to afford the desired product as a clear solid (140 mg, 60 %).  $\nu_{\text{max}}$  (neat): 3400, 2933, 2865, 1708, 1637, 1438, 1449  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.33-7.29 (m, 1H), 7.23-7.19 (m, 2H), 5.63 (br. s, 1H), 5.04 (s, 2H), 4.55-4.46 (m, 1H), 4.41 (t,  $J = 8.0$  Hz, 1H), 4.29 (t,  $J = 9.1$  Hz, 1H), 3.97 (dd,  $J = 5.1, 8.5$  Hz, 1H), 3.83 (dd,  $J = 5.2, 10.3$  Hz, 1H), 3.64-3.52 (m, 2H), 2.16-2.04 (m, 1H), 2.03-1.95 (m, 2H), 1.92-1.84 (m, 1H), 1.83-1.74 (m, 4H), 1.73-1.70 (m, 2H), 1.69-1.63 (m, 2H), 1.62-1.54 (m, 2H), 1.53-1.38 (m, 5H), 1.34-1.20 (m, 5H), 1.16-0.99 (m, 3H), 0.96-0.88 (m, 6H), 0.66 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  174.1, 169.1, 139.7, 135.3, 128.5, 126.3, 71.6, 71.5, 65.4, 58.1, 55.9, 55.1, 54.9, 51.0, 43.9, 42.6, 40.9, 40.3, 39.3, 37.5, 37.0, 35.6, 35.1, 34.2, 31.0, 30.5, 28.8, 28.7, 27.0, 23.5, 21.3, 18.7, 12.3. HRMS: exact mass calculated for  $[\text{M}-\text{H}]^-$  ( $\text{C}_{35}\text{H}_{49}\text{Cl}_2\text{N}_2\text{O}_5$ ) requires 647.3024  $m/z$ , found 647.3021  $m/z$ .

**3,5-Dichlorobenzyl** ((3*R*)-3-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamido)cyclopentyl)carbamate (**17**). Prepared according to General Procedure **E**

with ursodeoxycholic acid (149 mg, 0.38 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (306  $\mu$ L, 1.75 mmol, 5.0 equiv), HATU (145 mg, 0.38 mmol, 1.1 equiv), 3,5-dichlorobenzyl ((3*R*)-3-aminocyclopentyl)carbamate (100 mg, 0.35 mmol, 1.0 equiv) and DMF (10 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford the desired product as a clear solid (82 mg, 35%).  $\nu_{\max}$  (neat): 3301, 2931, 2968, 1697, 1654, 1420 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  7.29-7.26 (m, 1H), 7.22-7.18 (m, 2H), 5.03 (s, 2H), 4.50-4.41 (m, 1H), 3.72-3.60 (m, 1H), 3.60-3.42 (m, 4H), 3.30-3.20 (m, 1H), 2.25-2.12 (m, 2H), 2.08-2.01 (m, 1H), 1.99-1.93 (m, 1H), 1.89-1.71 (m, 6H), 1.68-1.61 (m, 2H), 1.58-1.53 (m, 2H), 1.49-1.38 (m, 6H), 1.31-1.19 (m, 5H), 1.14-0.97 (m, 3H), 0.96-0.87 (m, 6H), 0.63 (s, 3H). NH and 2  $\times$  OH not observed (exchangeable). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz):  $\delta$  173.8, 154.4, 140.2, 135.1, 128.2, 126.2, 71.31, 71.26, 65.4, 56.0, 55.1, 51.6, 49.4, 48.6, 44.6, 44.2, 43.8, 43.7, 42.6, 40.3, 39.4, 37.4, 37.2, 35.5, 35.0, 34.1, 33.5, 31.9, 30.9, 30.4, 28.8, 27.0, 23.5, 21.3, 18.6, 12.2. Rotameric mixture observed. HRMS: exact mass calculated for [M+NH<sub>4</sub>]<sup>+</sup> (C<sub>36</sub>H<sub>56</sub>Cl<sub>2</sub>N<sub>3</sub>O<sub>5</sub>) requires 680.3592 *m/z*, found 380.3588 *m/z*.

**3,5-Dichlorobenzyl ((3*S*)-3-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-**

**yl)pentanamido)cyclopentyl)carbamate (18).** Prepared according to General Procedure **E** with ursodeoxycholic acid (200 mg, 0.51 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (330  $\mu$ L, 0.19 mmol, 4.0 equiv), HATU (194 mg, 0.51 mmol, 1.1 equiv), 3,5-dichlorobenzyl 3,5-dichlorobenzyl (*S*)-3-aminopyrrolidine-1-carboxylate (136 mg, 0.47 mmol, 1.0 equiv) and DMF (10 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford the desired product as a clear solid (54 mg, 17%).  $\nu_{\max}$  (neat): 3450, 3415, 3030, 3963, 2940, 2862, 1725, 1637 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  7.32-7.29 (m, 1H), 7.25-7.21 (m, 2H), 5.91 (s, 1H), 5.04 (s, 2H), 4.46 (m, 1H), 3.72-3.61 (m, 3H), 3.62-3.54 (m, 2H), 3.54-3.46 (m, 2H), 3.25 (t, *J* = 12.1 Hz, 1H),

2.25-2.12 (m, 2H), 2.10-2.01 (m, 1H), 2.02-1.96 (m, 1H), 1.83-1.73 (m, 5H), 1.69-1.62 (m, 2H), 1.60-1.53 (m, 2H), 1.50-1.37 (m, 5H), 1.25-1.18 (m, 8H), 1.14-0.97 (m, 3H), 0.96-0.86 (m, 6H), 0.65 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  173.7, 154.5, 140.2, 135.2, 128.3, 126.2, 71.4, 65.4, 58.5, 55.9, 55.1, 51.7, 49.5, 48.7, 44.6, 44.2, 43.9, 42.6, 40.3, 39.4, 38.7, 37.4, 37.1, 35.5, 35.0, 34.2, 33.5, 32.0, 31.8, 30.9, 30.4, 28.8, 27.0, 23.5, 21.3, 18.6, 18.5, 12.2. Rotameric mixture observed. HRMS: exact mass calculated for  $[\text{M}+\text{Na}]^+$  ( $\text{C}_{36}\text{H}_{52}\text{Cl}_2\text{N}_2\text{NaO}_5$ ) requires 685.3145  $m/z$ , found 685.3129  $m/z$ .

**3,5-Dichlorobenzyl ((3*R*)-1-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)pyrrolidin-3-yl)carbamate (19).** Prepared according to General Procedure **E** with ursodeoxycholic acid (149 mg, 0.38 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (306  $\mu\text{L}$ , 1.75 mmol, 5.0 equiv), HATU (145 mg, 0.38 mmol, 1.1 equiv), 3,5-dichlorobenzyl (*R*)-pyrrolidin-3-ylcarbamate (100 mg, 0.35 mmol, 1.0 equiv) and DMF (10 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to afford the desired product as a clear solid (36 mg, 15%).  $\nu_{\text{max}}$  (neat): 3298, 2933, 2868, 1706, 1624, 1436  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz):  $\delta$  7.33-7.30 (m, 1H), 7.24-7.21 (m, 2H), 5.07-5.02 (m, 2H), 5.02-4.97 (m, 1H), 4.36-4.21 (m, 1H), 3.78-3.65 (m, 1H), 3.62-3.50 (m, 4H), 3.45-3.31 (m, 1H), 2.33-2.22 (m, 1H), 2.22-2.09 (m, 1H), 2.04-1.97 (m, 1H), 1.93-1.86 (m, 1H), 1.85-1.75 (m, 4H), 1.71-1.63 (m, 2H), 1.63-1.55 (m, 4H), 1.53-1.47 (m, 2H), 1.47-1.41 (m, 4H), 1.39-1.18 (m, 6H), 1.17-0.98 (m, 3H), 0.97-0.90 (m, 6H), 0.67 (s, 3H). NH not observed.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz):  $\delta$  172.8, 172.7, 155.6, 155.5, 139.9, 139.8, 135.24, 135.20, 128.4, 128.3, 126.23, 126.20, 71.5, 71.4, 65.22, 65.15, 55.9, 55.1, 52.5, 51.5, 51.0, 50.8, 50.0, 44.7, 43.85, 43.82, 43.78, 42.6, 40.3, 39.3, 37.4, 37.1, 35.6, 35.5, 35.1, 34.2, 32.4, 31.8, 31.4, 31.0, 30.4, 28.82, 28.78, 27.0, 23.5, 21.3, 18.7, 12.3. Rotameric mixture observed. HRMS: exact mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{36}\text{H}_{53}\text{Cl}_2\text{N}_2\text{O}_5$ ) requires 663.3326  $m/z$ , found 663.3323  $m/z$ .

**3,5-Dichlorobenzyl ((3*S*)-1-((4*R*)-4-((3*R*,7*S*,10*S*,13*R*)-3,7-dihydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoyl)pyrrolidin-3-yl)carbamate (20).**

Prepared according to General Procedure **E** with ursodeoxycholic acid (200 mg, 0.51 mmol, 1.1 equiv), *N,N*-diisopropylethylamine (330  $\mu$ L, 0.19 mmol, 4.0 equiv), HATU (194 mg, 0.51 mmol, 1.1 equiv), 3,5-dichlorobenzyl 3,5-dichlorobenzyl (*S*)-pyrrolidin-3-ylcarbamate (136 mg, 0.47 mmol, 1.0 equiv) and DMF (10 mL). The crude material was subjected to the purification outlined in the General Procedure (silica gel, 0-10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford the desired product as a clear solid (56 mg, 18%).  $\nu_{\text{max}}$  (neat): 3294, 2931, 2866, 1708, 1626, 1436 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz):  $\delta$  7.32-7.29 (m, 1H), 7.26-7.21 (m, 2H), 5.08-5.02 (m, 2H), 4.34-4.20 (m, 1H), 3.77-3.65 (m, 1H), 3.65-3.48 (m, 4H), 3.45-3.29 (m, 1H), 2.33-2.23 (m, 1H), 2.20-2.11 (m, 1H), 2.06-1.97 (m, 1H), 1.95-1.87 (m, 1H), 1.84-1.74 (m, 4H), 1.71-1.64 (m, 2H), 1.62-1.53 (m, 5H), 1.52-1.41 (m, 6H), 1.37-1.30 (m, 2H), 1.30-1.21 (m, 4H), 1.17-1.11 (m, 1H), 1.09-0.98 (m, 2H), 0.97-0.91 (m, 6H), 0.67 (d, *J* = 2.0 Hz, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz):  $\delta$  172.8, 172.7, 155.4, 139.8, 139.7, 135.3, 128.5, 126.3, 71.6, 71.5, 65.3, 55.9, 55.1, 52.6, 51.5, 51.1, 50.0, 44.6, 43.9, 43.8, 42.6, 40.3, 39.3, 37.5, 37.0, 35.6, 35.1, 34.2, 32.5, 31.8, 31.4, 31.0, 30.5, 29.8, 28.8, 27.1, 23.5, 21.3, 18.8, 12.3. Rotameric mixture observed. HRMS: exact mass calculated for [M+Na]<sup>+</sup> (C<sub>36</sub>H<sub>52</sub>Cl<sub>2</sub>N<sub>2</sub>NaO<sub>5</sub>) requires 685.3145 *m/z*, found 685.3113 *m/z*.

**Protein production and crystallization.** ATX from rat was produced in HEK293 Flp-In cells as described previously.<sup>16</sup> For co-crystallization of rATX with **3**, **11**, **5**, and **17**, rATX (3.5 mg mL<sup>-1</sup>) was first mixed in 10 molar excess with the compound and incubated for 30 min. In all cases, the best crystals were obtained at room temperature (293 K) in a 24-well optimization screen (18-20% PEG 3350, 0.1-0.4 M NaSCN and 0.1-0.4 M NH<sub>4</sub>I) by mixing 1  $\mu$ L of protein:compound solution and 1  $\mu$ L of reservoir solution. All crystals were vitrified in cryoprotectant, which consisted of reservoir solution with the addition of 20% (v/v) glycerol.

**Crystallographic data and methods.** The X-ray diffraction data for the ATX-inhibitor complexes were collected at SLS on beamlines PXIII (**3**, **11**, and **5**) and PXI (**17**) at 100K. The crystallographic diffraction data for the ATX-inhibitor complexes were recorded on a PILATUS 2M-F detector for **3**, **11**, and **5** to a resolution of 1.95, 2.4, and 2.1 Å, respectively and on the EIGER 16M detector for **17** to 2.1 Å. All data were processed and integrated with XDS.<sup>17</sup> **17** was processed on site using the SLS automated processing pipeline and scaled with AIMLESS.<sup>18</sup> The structures were determined by molecular replacement using PHASER<sup>19</sup> with the structure of ATX (PDB entry 2XR9) as the search model. Model building and subsequent refinement were performed iteratively with COOT<sup>20</sup> REFMAC5<sup>21</sup> and PDB\_REDO.<sup>22</sup> Structure validation was carried out by MolProbity. The structure models and experimental diffraction data were deposited at the PDB under codes 5MOS, 5MOM, 5MOD, 5MOE). Crystallographic data and refinement details are available in Table 2.

Table 2. Crystallography Details

|  | 3                | 5                    | 11                   | 17                   |
|--|------------------|----------------------|----------------------|----------------------|
| Data Collection                          |                  |                      |                      |                      |
| Wavelength (Å)                           | 1.0000           | 0.9801               | 0.9801               | 1.0000               |
| Resolution (Å)                           | 1.95             | 2.4                  | 2.1                  | 2.1                  |
| Space Group                              | P 1              | P 1 2 <sub>1</sub> 1 | P 1 2 <sub>1</sub> 1 | P 1 2 <sub>1</sub> 1 |
| Unit Cell a, b, c (Å)                    | 53.6, 61.7, 63.7 | 62.9, 88.0, 77.4     | 62.8, 88.9, 77.3     | 62.9, 89.3, 77.2     |
| α, β, γ (°)                              | 103, 98, 93      | α=β=γ=90             | α=β=γ=90             | α=β=γ=90             |
| CC <sub>1/2</sub>                        | 0.996 (0.481)    | 0.998 (0.677)        | 0.998 (0.568)        | 0.998 (0.487)        |
| R <sub>merge</sub>                       | 0.044 (0.812)    | 0.025 (0.921)        | 0.027 (1.148)        | 0.026 (0.766)        |
| I/σI                                     | 18.9 (1.5)       | 41.9 (1.6)           | 32.6 (1.2)           | 19.1 (1.6)           |
| Completeness (%)                         | 98.8 (96.6)      | 98.3 (99.6)          | 97.1 (97.9)          | 89.2 (95.4)          |
| Redundancy                               | 3.9 (3.8)        | 4.5 (4.5)            | 3.8 (4.0)            | 27 (2.5)             |
| Refinement                               |                  |                      |                      |                      |
| No. atoms                                | 6700             | 6451                 | 6592                 | 6598                 |
| Protein                                  | 6275             | 6251                 | 6275                 | 6242                 |
| Ligand/Metal/Glycan                      | 186              | 172                  | 215                  | 256                  |
| Water/Iodine                             | 239              | 28                   | 102                  | 100                  |
| TLS groups                               | 1                | 1                    | 1                    | 1                    |
| R <sub>work</sub> /R <sub>free</sub> (%) | 18.1/22.3        | 22.4/27.0            | 23.8/28.6            | 20.1/24.9            |
| Validation                               |                  |                      |                      |                      |
| Rmsd/rmsZ bond lengths (Å)               | 0.012            | 0.008                | 0.007                | 0.011                |
| Rmsd/rmsZ bond angles (°)                | 1.631            | 1.377                | 1.249                | 1.515                |
| Ramachandran Preferred/ outliers         | 98/0             | 95/0                 | 97/0                 | 96/0                 |
| Sidechain rotamers Preferred             | 94               | 94                   | 96                   | 95                   |
| MolProbity/Clash score (%ile)            | 100/100          | 100/100              | 100/100              | 100/100              |

High Resolution shell in parentheses

**Biochemical assays and modeling of kinetic data.** ATX lysoPLD activity was measured by choline release from LPC.<sup>23</sup> 20 nM human ATX (prepared from HEK 293 Flp-In cells) was incubated 150 mM LPC (18:1) in a final volume of 100  $\mu$ L buffer containing 50 mM Tris, pH 7.4, 0.01% Triton X-100, 50 mM  $\text{CaCl}_2$ , 1 U  $\text{mL}^{-1}$  horseradish peroxidase, 2 mM homovanillic acid (HVA). The relative amount of released choline was measured by HVA fluorescence in a 96-well plate (Corning). Fluorescence intensity was determined at ex/em 320/450 nm every 30 s for 90 min with a Fluorostart plate reader (BMG Labtech). Data analysis was performed using GraphPad Prism. The experiments for determining  $\text{IC}_{50}$  values for the different inhibitors were performed by serial threefold dilutions; for each inhibitor concentration, the velocity of the reaction was monitored as a function of time in the lysoPLD assay as described above. The linear part of the reaction velocity was visually estimated (typically between 15 min and 60 min) and the observed fluorescence signal (described above) as a function of time was modeled for all inhibitor concentrations simultaneously using the formula:

$$F_t = F_0 + \left( \frac{v_{\max} - v_{\min}}{(1 + C_{\text{inh}}/\text{IC}_{50})} + v_{\min} \right) t$$

where  $F_t$  is the observed fluorescence signal relative to the choline product at each measurement,  $v_{\max}$  and  $v_{\min}$  are the fitted values for the minimum and maximum relative velocity and  $c_{\text{inh}}$  is the corresponding inhibitor concentration. To identify the mode of inhibition of ATX by **17**, we used regression analysis tools within the GraphPad Prism software to determine the value of  $\alpha$  using the built-in formula for the mixed inhibition model. The values of all derived parameters indicate mean values as obtained by nonlinear regression analysis; triplicate data were used as separate observations during the fit;  $\pm$  values indicate the standard error of the mean.

**Cell-based assays.** BJEH fibroblasts were seeded in 6-well tissue culture plates in DMEM (GIBCO, Life Technologies) containing 10% FCS (Hyclone, Thermo Scientific), 2 mM glutamine, 100  $\text{U mL}^{-1}$  penicillin and 100  $\text{mg mL}^{-1}$  streptomycin (GIBCO, Life Technologies). When  $\pm$  60-90 confluent, they were serum starved (DMEM with glutamine and pen/strep,

without FCS) for 16 hours. hATX (10 nM final concentration) was incubated with inhibitor for 30 min in serum free medium (total volume 1 mL). Medium from the 6-well plates was removed and replaced with 1 mL of ATX-inhibitor (**17**) mixture. Cells were stimulated for 15 min, medium was removed and plates were immediately frozen on dry ice and stored at -80 °C. Cells were lysed in the plates on ice for  $\pm$  10 min (lysis buffer: RIPA, containing sodium fluoride, orthovanadate and protease inhibitors). Lysates were centrifuged for 30 minutes at 10,000 rpm to remove cell debris and were taken up in loading buffer containing DTT and boiled for 10 min at 95°C. Samples were loaded on a pre-cast gel (Bis-Tris Invitrogen 4-12%) and separated by gel electrophoresis. The gel was transferred by western blotting to a nitrocellulose membrane which was blocked in milk (to reduce non-specific binding) before the membrane was probed with the desired primary antibodies overnight (in this case p-AKT and total AKT). The membrane was washed 3  $\times$  in PBS-tween and probed with a HRP-conjugated anti-Rabbit antibody for 1 hour. Washed 3  $\times$  and developed using ECL reagent using a BioRad chemidoc imaging system.

**Mouse experiments.** ATX inhibitors were dissolved at 50 mgmL<sup>-1</sup> in vehicle (Hydroxypropyl Cellulose 2% / Tween 80 0.1%) and administered intraperitoneally at 10 mgkg<sup>-1</sup>. Blood was collected after 8 hours, plasma isolated and levels of 8 abundant LPA species determined by HPLC electrospray ionization tandem mass spectrometry as reported previously.<sup>15</sup> Protocols and procedures for animal studies conformed to current US Public Health Service policy and were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

### Abbreviations

Autotaxin (ATX); Human transformed skin fibroblasts (BJEH); Ectonucleotide pyrophosphatase 2 (ENPP2); Lysophosphatidic acid (LPA); Lysophosphatidyl choline (LPC); phosphor-serine/threonine protein kinase (pAKT); Structure activity relationship (SAR); Somatomedin- $\beta$  (SMB); Tauroursodeoxycholic acid (TUDCA); Ursodeoxycholic acid (UDCA).



## Associated Content

Supporting information. Spectral data for all compounds, preparative details for diamine monomers and crystallographic refinement data (PDF).

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## Accession Codes

PDB codes for the structures presented are 5MOE (**3**), 5MOS (**5**), 5MOD (**11**) and 5MOM (**17**). Authors will release the atomic coordinates and experimental data upon article publication.

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*Tunnel*

*Lipophilic pocket*

- competitive ATX inhibitor (nM)
- identified through structure based design
- modulates LPA synthesis *in vivo*