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# Autotaxin – A Target for the Treatment of Drug-Resistant Ovarian Cancer?

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## 1. Introduction

It has been known for a number of years that many patients with ovarian cancer suffer an accumulation of ascites fluid that contains a factor which supports the intraperitoneal growth of ovarian cancer cells (Mills et al. 1990). Following from the identification of lysophosphatidic acid (LPA) as a major growth factor in serum (van Corven et al. 1989), LPA was identified as the major “ovarian cancer activating factor” in ascites fluid (Xu et al. 1995). LPA was shown to accumulate to high concentrations (up to 80  $\mu$ M) in ascites fluid. Since then, numerous publications have demonstrated the role of LPA in several biological processes relevant to cancer including cell migration and invasion, inhibition of apoptosis and senescence, angiogenesis and chemoresistance. Increases in plasma LPA are also being considered as a diagnostic biomarker of ovarian cancer (e.g. (Bese et al. 2010)). It is perhaps surprising then, that compounds interfering with this pathway have made slow progress to the clinic. Part of the reason for this likely reflects the complexity of the LPA signalling pathway. However, recent work has delineated many of the enzymes and receptors involved in regulating the LPA signalling pathways, revealing complexity in different LPA species, in the pathways involved in the metabolism of LPA, in LPA receptors and finally in the (patho)physiological responses to LPA. An understanding of how these pathways are deregulated in ovarian cancer has begun to suggest potential targets for the development of therapeutic drugs. One such target is autotaxin, an enzyme involved in the synthesis of LPA. Recently, several crystal structures of autotaxin have been solved, and these provide powerful tools to aid the development of autotaxin inhibitors. However, to fully appreciate the potential of autotaxin as a drug target, we first review LPA signalling pathways.

## 2. The LPA signalling pathway

### 2.1 Complexity in LPA

LPA (Fig. 1) itself provides a first example of complexity in this pathway, as it comprises a family of molecules. In general, LPA consists of a glycerol moiety linked as an ester to phosphate and fatty acid moieties. However, LPA molecules may differ in the length and the degree of unsaturation of the fatty acid, and the fatty acid may be attached to the *sn*1 or

*sn*2 positions on the glycerol. In some cases, the fatty acid is replaced by an alkyl chain attached via an ether linkage. The phosphate may be attached to both the second and third glycerol hydroxyl groups, forming a cyclophosphate.

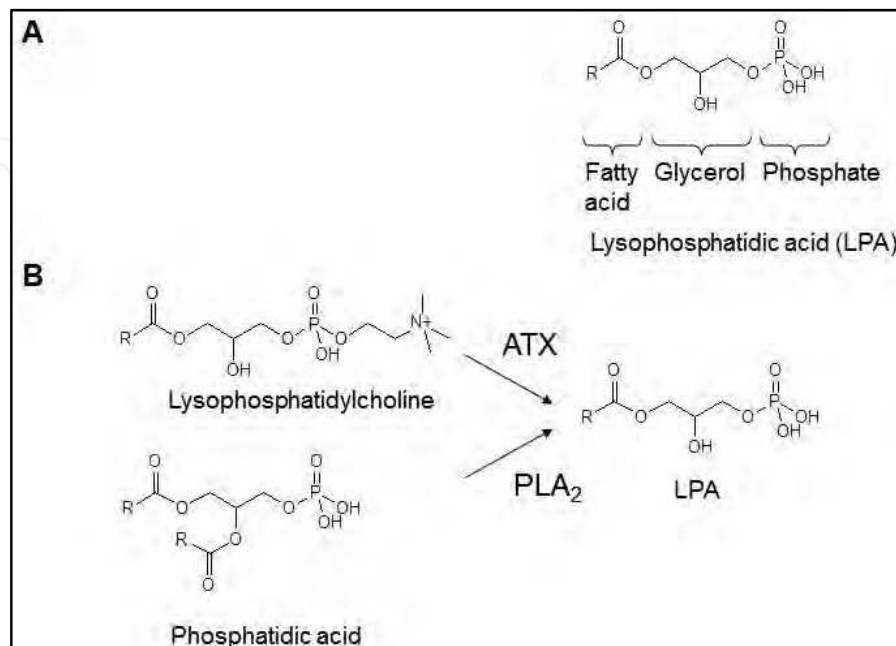


Fig. 1. **A** Lysophosphatidic acid is comprised of fatty acid, glycerol and phosphate moieties. **B**. Principle routes to the biosynthesis of LPA. ATX, autotaxin; PLA<sub>2</sub>, phospholipase A<sub>2</sub>. Note that autotaxin behaves as a phospholipase D that uses *lyso* substrates, i.e. those lacking one fatty acid attached to the glycerol.

## 2.2 Complexity in the synthesis of LPA

There are a number of enzymes which can catalyze the synthesis of LPA in ovarian cancer and it is becoming more clear which of these contribute to the accumulation of LPA in ovarian cancer. LPA is synthesized both intracellularly and extracellularly, by ovarian cancer cells as well as mesothelial cells. Autotaxin is a secreted phospholipase that catalyses the hydrolysis of lysophosphatidyl choline to produce LPA and choline (fig. 2). Increased expression of autotaxin is observed in several cancers including renal (Stassar et al. 2001), thyroid (Kehlen et al. 2004), glioblastoma (Hoelzinger et al. 2005; Kishi et al. 2006), follicular lymphoma (Masuda et al. 2008), hepatic (Wu et al. 2010), prostate (Nouh et al. 2009) and pancreatic cancer (Nakai et al. 2011). Autotaxin expression is also increased in chemoresistant ovarian cancer compared to chemosensitive disease (Jazaeri et al. 2005). Ectopic expression of autotaxin in mammary epithelium is sufficient to cause high frequency breast cancer (Liu et al. 2009). Together these observations point to role for autotaxin in several cancer types. The elevated levels of LPA observed in ascites obtained from patients with ovarian cancer suggests a role in ovarian cancer. The increase in LPA levels are accompanied by elevated LPC, the substrate of autotaxin (Liu et al. 2009). Autotaxin itself is also present in ascites fluid (Tokumura et al. 2007). Although transgenic mice lacking autotaxin die as embryos, heterozygotes with one functional allele encoding autotaxin show a 50% reduction in plasma LPA (Tanaka et al. 2006), suggesting that autotaxin is the enzyme primarily responsible for the synthesis of LPA in plasma. However,

it remains a possibility that autotaxin is not the enzyme responsible for the accumulation of LPA observed in ovarian cancer. In addition to autotaxin, extracellular LPA could potentially also be derived from the hydrolysis of phosphatidic acid by secreted phospholipase A<sub>2</sub> (sPLA<sub>2</sub>).

A number of intracellular enzymes including glycerol 3-phosphate fatty acid transferase (GPFAT) and phospholipase D (PLD) and phospholipase A<sub>2</sub> may also contribute to the production of LPA at the cell membrane. It is possible that intracellular enzymes contribute to the accumulation of extracellular LPA. Although GPFAT has not received much attention in ovarian cancer, PLD<sub>2</sub> has recently been shown to contribute to EGF-induced LPA production (Snider et al. 2010). Other potential sources of LPA include intracellular isoforms of phospholipase A<sub>2</sub> which use phosphatidic acid as a substrate (Fig. 1). cPLA<sub>2</sub> is a calcium-dependant phospholipase implicated in cell migration whereas iPLA<sub>2</sub> is a calcium-independent phospholipase. Which cells are the potential sources of LPA? Platelets have previously been shown to be an important source of LPA in serum (Boucharaba et al. 2004), and Xu and co-workers have clearly shown in a murine model of ovarian cancer that both host and tumor cells contribute to the formation of LPA in ascites and this is catalysed by iPLA<sub>2</sub> (Li et al. 2010). Peritoneal mesothelial cells are one potential source of extracellular LPA produced by PLA<sub>2</sub> (Ren et al. 2006).

If both host and tumor cells contribute to the accumulation of peritoneal LPA, and there are several pathways capable of contributing to the formation of LPA, it seems reasonable to ask whether animal models accurately reflect clinical reality. In xenograft studies it is common to implant a human tumor cell into a murine host. Is the relative contribution of different LPA biosynthetic pathways in xenograft studies quantitatively similar to that observed in ovarian cancer? This is important because the relative contribution of, e.g., autotaxin and iPLA<sub>2</sub> to the generation of LPA will likely influence the success of inhibitors of these individual enzymes when used in patients. Thus, we consider that although preclinical experiments may continue to shed light on validity of the different LPA biosynthetic pathways as drug targets, a definitive answer will only be provided by clinical studies.

The concentration of LPA in ascites fluid is controlled by its rate of elimination as well as its rate of synthesis. It is important, therefore, to consider also pathways of LPA catabolism. Two lipid phosphatases, LPP1 and LPP3, have been implicated in the hydrolysis of LPA. Importantly, LPP1 shows reduced expression in ovarian cancer cells, suggesting that this might contribute to increased levels of LPA (Tanyi et al. 2003; Tanyi et al. 2003). Correspondingly, expression of these genes has been shown to inhibit several of the responses ascribed to LPA, for example colony formation and cell migration. Understanding the pathways that regulate the expression of these phosphatases is important, as it might provide targets which can be used to increase the expression of LPP1 or LPP3 and so develop drugs to increase LPA catabolism.

It is also worth considering how the expression of LPA anabolic and catabolic enzymes might vary between patients as this may influence the design of clinical trials. Although we await further data to address this, it is worth considering the potential impact on the clinical use of drugs regulating the LPA pathway. It seems that it will be appropriate to select patients most likely to benefit from a particular enzyme inhibitor taking into account which biosynthetic pathways are deregulated. For example, PLD<sub>2</sub> has been implicated in EGF-driven LPA production (Snider et al. 2010), so patients whose tumors are driven by the EGF pathway may be more dependent on PLD<sub>2</sub> than other LPA producing enzymes. Similarly, VEGF regulates autotaxin production (see below), so tumors in which VEGF production is

substantially elevated may be more dependent on autotaxin for LPA production. The expression level of LPP1 or LPP3 may also influence the response to drugs inhibiting the production of LPA. We speculate that inter-patient variability in the enzymes catalysing LPA catabolism may lead to different response to drugs which inhibit LPA synthesis and evaluating the extent of any clinical variation may prove to be important.

### 2.3 Complexity in LPA receptors

Two classes of cell surface receptors for LPA have been described, all of which are G-protein coupled receptors (Tigyi 2010). The first of these classes comprise the receptors LPA<sub>1</sub>, LPA<sub>2</sub> and LPA<sub>3</sub>. These are closely related and form part of the EDG (endothelial differentiation gene) family of receptors which also includes receptors for the bioactive lipid sphingosine 1-phosphate (S1P). At high ( $\mu\text{M}$ ) concentrations, LPA may also bind to S1P receptors. A second set of LPA receptors are more closely related to purinergic receptors including LPA<sub>4</sub> (also known as P2Y<sub>9</sub>), LPA<sub>5</sub> (GPR95). There are several additional receptors that are also reported to respond to LPA including GPR35, GPR87, P2Y<sub>5</sub> and P2Y<sub>10</sub> and further characterization of these is on-going. Clearly, it is important to consider which of these receptors should be exploited as drug targets in ovarian cancer.

LPA<sub>2</sub> and LPA<sub>3</sub> appear to promote ovarian tumorigenesis. The expression of LPA<sub>2</sub> and LPA<sub>3</sub> is increased in ovarian cancer, (Fang et al. 2002; Wang et al. 2007; Murph et al. 2008) and over-expression of these receptors in Sk-Ov-3 cells promotes growth of primary tumors and metastasis (Yu et al. 2008). In clinical samples, expression of LPA<sub>2</sub> and LPA<sub>3</sub> correlates with tumor stage (Wang et al. 2007). In contrast, the expression of LPA<sub>1</sub> is decreased in ovarian cancer and expression of LPA<sub>1</sub> promotes apoptosis (Furui et al. 1999). These observations are important from a therapeutic perspective, because it suggest that ovarian cancer patients might benefit from a drug which is an LPA<sub>2</sub>, LPA<sub>3</sub> antagonist but it may be preferable that such a drug does not bind with high affinity to LPA<sub>1</sub>. However, it should be noted that the growth inhibitory properties of LPA<sub>1</sub> were found to be independent of LPA (Furui et al. 1999) and encouraging results have already been obtained with a pan-LPA receptor antagonist in xenograft studies (Zhang et al. 2009). We already have substantial experience developing (non-oncological) drugs using G-protein coupled receptors as drug targets, suggesting this may be a fruitful avenue for therapeutic research.

As well as binding to cell surface receptors, LPA has been proposed to activate the nuclear hormone receptor PPAR $\gamma$ . These intracellular receptors function as transcription factors and drive the expression of genes involved in diverse physiological responses including glucose and lipid metabolism, inflammatory response and apoptosis. PPAR $\gamma$  is over-expressed in ovarian cancer (Zhang et al. 2005) and its expression is associated with a poor response to chemotherapy and shortened survival (Davidson et al. 2009). Although this might lead to the hypothesis that activation of PPAR $\gamma$  by LPA is tumorigenic, confusingly synthetic PPAR agonists (the “glitazones”) inhibit the proliferation of ovarian cancer cells and induce apoptosis (Yang et al. 2007). Glitazones also display synergistic activity with platinum chemotherapy through down-regulation of metallothionines involved in the detoxification of platinum (Girnun et al. 2007). Thus, the contribution of the activation of PPAR $\gamma$  by LPA to ovarian tumorigenesis remains to be further clarified.

Finally, it has also been pointed out that LPA binds to a number of intracellular cytoskeletal proteins (Tigyi 2010) possibly reflecting an intracellular role for LPA in regulating cell migration.

### 3. Physiological and pathophysiological functions of LPA

#### 3.1 LPA/autotaxin and migration and invasion

LPA has several well characterized effects upon ovarian cancer cell migration and invasion. Firstly, activation of Src kinase by LPA leads to the breakdown of cell-cell junctions, promoting cell scattering (Huang et al. 2008). The breakdown of cell junctions is facilitated by activation uPA (urokinase plasminogen activator) by LPA, which leads to proteolysis of E-cadherin. Secondly, LPA triggers cytoskeletal reorganization (Do et al. 2007; Kim et al. 2011) and reorganization of cell contacts with the extracellular matrix which promotes cell motility (Sawada et al. 2002; Bian et al. 2004; Bian et al. 2006). Thirdly, LPA induces the expression of several proteases including uPA (Pustilnik et al. 1999; Li et al. 2005), MMP1 (Wang et al. 2011), MMP2 (Fishman et al. 2001) MMP7 and MMP9 (Park et al. 2011) which contribute to the breakdown of extracellular matrix allowing invasion through basement membrane. In addition, LPA decreases expression of TIMP metalloprotease inhibitors (Sengupta et al. 2007), thereby potentiating the effect of activation of proteases.

Although the role of autotaxin in migration and invasion has not yet been studied in ovarian cancer to the same level of detail as in other cancers, the role of autotaxin in these processes is well founded. Indeed, autotaxin was first identified through its activity as an autocrine motility factor (Stracke et al. 1992) and integrin  $\alpha_6\beta_4$ , which is associated with an invasive phenotype, can increase the expression of autotaxin (Chen and O'Connor 2005). Autotaxin and LPA promote the expression of the extracellular matrix protein osteopontin which promotes migration (Zhang et al. 2011). Autotaxin activates the small G-proteins cdc42 and Rac (Jung et al. 2002; Hoelzinger et al. 2008; Harper et al. 2010) and focal adhesion kinase (Jung et al. 2002), proteins which are key regulators of cell motility. More direct evidence comes from the observation that knockdown of autotaxin inhibits cell migration in several cancer types (Kishi et al. 2006; Gaetano et al. 2009; Harper et al. 2010) and over-expression of autotaxin increases motility (Kishi et al. 2006; Harper et al. 2010). Autotaxin regulates the formation of invadopodia (Harper et al. 2010) and induces the expression of uPA (Lee et al. 2006) and MMP3 (Haga et al. 2009). Correspondingly, knockdown of autotaxin inhibits invasion (Hoelzinger et al. 2008) while over-expression promotes invasion (Nam et al. 2000; Yang et al. 2002). Finally, autotaxin promotes osteolytic bone metastases derived from breast cancer cells (David et al. 2010). Taken together, these observations suggest that LPA and autotaxin are likely to promote an invasive phenotype in ovarian cancer cells. We discuss below the therapeutic implications of these observations.

#### 3.2 LPA/autotaxin and a supportive microenvironment

LPA contributes to providing a microenvironment that is conducive to tumor growth. It does this in part by suppressing apoptosis and senescence. LPA is itself a growth factor (van Corven et al. 1989) for several cell types. It stimulates the growth of cultures of ovarian cancer cells (Xu et al. 1995; Hu et al. 2003) by several pathways (Hurst and Hooks 2009). It also induces the expression of the growth factor Gro $\alpha$  (Lee et al. 2006). Finally, iPLA2, one of the enzymes involved in the synthesis of LPA, can promote cell cycle progression in the absence of exogenous growth factors (Song et al. 2007).

LPA induces the production of the major angiogenic factor VEGF by ovarian cancer cells (Hu et al. 2001) and mesenchymal stem cells (Jeon et al. 2010). LPA also increases VEGF receptor expression on endothelial cells. The effect of LPA is apparently amplified by VEGF-

induced expression of autotaxin by ovarian cancer cells (Ptaszynska et al. 2008) and endothelial cells (Ptaszynska et al. 2010) thereby potentiating LPA production. LPA also promotes the expression of other pro-angiogenic factors including IL-8 by tumor cells and SDF-1 by mesenchymal stem cells (Jeon et al. 2010). These observations suggest a key role for autotaxin and LPA in ovarian cancer driven angiogenesis and have led to the suggestion that autotaxin may also be a therapeutic target for inhibiting angiogenesis (Ptaszynska et al. 2010). In addition to its role in angiogenesis VEGF has also been implicated in LPA induced invasion (So et al. 2005; Wang et al. 2009; Wang et al. 2011).

### 3.3 LPA/autotaxin and inhibition of apoptosis and chemoresistance

The potential contribution of LPA to resistance to chemotherapy is of considerable therapeutic significance. Patients with ovarian cancer often receive chemotherapy comprising a taxane and a platinum-based compound, often paclitaxel and carboplatin. Although these drugs are initially effective, many patients eventually relapse with a disease that has become resistant to chemotherapy. Thus, a key reason that approximately 30% of patients diagnosed with ovarian cancer survive only 5-years post-diagnosis is the development of drug resistance. Understanding the molecular basis of drug resistance and developing drugs which restore drug sensitivity is one strategy to improve the treatment of ovarian cancer.

LPA causes the translocation of the pro-apoptotic receptor Fas from the cell surface, making tumor cells less responsive to stimuli that activate the extrinsic apoptosis pathway (Meng et al. 2005). Fas activates an intracellular caspase protease cascade to drive apoptosis. cFLIP is an inhibitor of caspase-8 activation, and the increased expression of cFLIP that is induced by LPA further contributes to suppression of apoptosis by LPA (Kang et al. 2004). At the same time, LPA induces the expression of Fas ligand (FasL) on tumor cells, and this promotes apoptosis of lymphocytes (Meng et al. 2004; Meng et al. 2005) presumably allowing tumor cells to avoid immune surveillance. LPA also increases the expression of the survival factor GEP (Kamrava et al. 2005). LPA inhibits the intrinsic apoptosis pathway by promoting phosphorylation of the pro-apoptotic protein BAD (Kang et al. 2004), which prevents BAD from promoting apoptosis through activation of Bak and Bax and permeabilization of the mitochondrial outer membrane. These observations suggest that LPA can regulate both the intrinsic and extrinsic apoptosis pathways, underlining the importance of this pathway as a therapeutic target.

In addition to LPA, there is evidence directly linking autotaxin to cell survival. Expression of autotaxin suppresses apoptosis in response to serum starvation (Song et al. 2005). LPA has been shown to activate the PI 3-kinase/Akt pathway in several cell types, including in ovarian cancer cells (Baudhuin et al. 2002). This pathway is a well described cell survival pathway and contributes to LPA suppressing both the extrinsic and the intrinsic apoptosis pathways (Kang et al. 2004). Similarly, inhibition of apoptosis by autotaxin is dependent on the PI 3-kinase pathway (Song et al. 2005).

As well as inhibition of apoptosis, one of the hallmarks of cancer is the avoidance of senescence. LPA suppresses p53-dependant replicative senescence (Kortlever et al. 2008), at least in part through induction of telomerase (Bermudez et al. 2007; Yang et al. 2008).

Along with many other chemotherapeutic agents, carboplatin and paclitaxel induce apoptosis. It seems reasonable to presume that the ability of autotaxin and LPA to suppress

apoptosis contributes to resistance to paclitaxel and carboplatin. As these drugs are the cornerstone of ovarian cancer chemotherapy, the potential of the LPA pathway as a therapeutic target is again underlined. Early work demonstrated that LPA confers resistance to cisplatin (Frankel and Mills 1996) and this has also been observed in colon cancer cells (Sun et al. 2009). We conducted a screen to identify genes that confer resistance to carboplatin, and one of the hits identified in that screen was autotaxin. Expression of autotaxin delayed apoptosis induced by carboplatin, while apoptosis was accelerated after inhibition of autotaxin by either siRNA or with a small molecule inhibitor (Vidot et al. 2010). More recently, LPA and autotaxin have been shown to confer resistance of breast and melanoma cancer cells to paclitaxel (Samadi et al. 2009). Resistance to paclitaxel depends on PI 3-kinase, presumably reflecting the role of PI 3-kinase downstream of LPA in survival signalling that was noted above. Remarkably, resistance to paclitaxel conferred by LPA by restores normal spindle function in cells exposed to paclitaxel and the cells escape M-phase arrest (Samadi et al. 2011). The LPA<sub>2</sub> receptor is one candidate for mediating chemoresistance, because LPA<sub>2</sub><sup>-/-</sup> mice exhibit increased radiation-induced apoptosis (Deng et al. 2007). Thus, there is direct evidence linking autotaxin to resistance to both chemotherapeutic agents used to treat ovarian cancer.

Other proteins in the LPA pathway may also contribute to chemoresistance. RGS proteins (Regulator of G-protein signalling) attenuate signalling by LPA receptors by increasing the GTPase activity of G-proteins that are activated by LPA receptors (Hurst et al. 2008). Expression of several RGS proteins is decreased in ovarian cancer cell lines that are resistant to cisplatin (Hooks et al. 2010). Knockdown of expression of two RGS protein, RGS10 and RGS17, causes a 2-3 fold reduction in the potency but a striking 6-fold reduction in cisplatin potency is observed when the expression of both RGS proteins is inhibited. This suggests that loss of expression of RGS proteins, leading to increased activity of LPA receptor signalling through G-proteins, may contribute to resistance to chemotherapy.

In addition to inhibiting apoptosis through G-protein signalling, the LPA<sub>2</sub> receptor also regulates the pro-apoptotic protein Siva-1. Activation of p53 following DNA damage increases the expression of pro-apoptotic Siva-1 and this contributes to cisplatin-induced apoptosis (Barkinge et al. 2009), as well apoptosis induced by ultraviolet light (Chu et al. 2004). LPA causes ubiquitination and turnover of Siva-1 and this contributes to suppression of apoptosis by LPA (Lin et al. 2007). This may be mediated by the LPA<sub>2</sub> receptor. LPA<sub>2</sub> is distinct from other LPA receptors in containing zinc finger and a C-terminal PDZ binding motifs. These motifs serve to recruit NHERF2 and TRIP6, which form a ternary complex with Siva-1. Both NHERF2 and TRIP6 are required for LPA to confer resistance to cisplatin (E et al. 2009). But how does Siva-1 induce apoptosis? In part, this probably reflects inhibition of the cell survival driven by the transcription factor NFκB. Intriguingly, Siva-1 can also inhibit Bcl-X<sub>L</sub>, a member of the anti-apoptotic Bcl-2 family proteins that suppress activation of Bak and Bax in the intrinsic apoptosis pathway (Xue et al. 2002). We have shown previously that inhibition of Bcl-X<sub>L</sub> increases sensitivity to carboplatin (Witham et al. 2007). Together with our observation that autotaxin confers resistance to carboplatin (Vidot et al. 2010), these data suggest that autotaxin may confer resistance to carboplatin by suppressing the intrinsic apoptosis pathway (fig. 2). As we discuss below, this predicts that autotaxin inhibitors may be useful in the treatment of drug-resistant ovarian cancer.



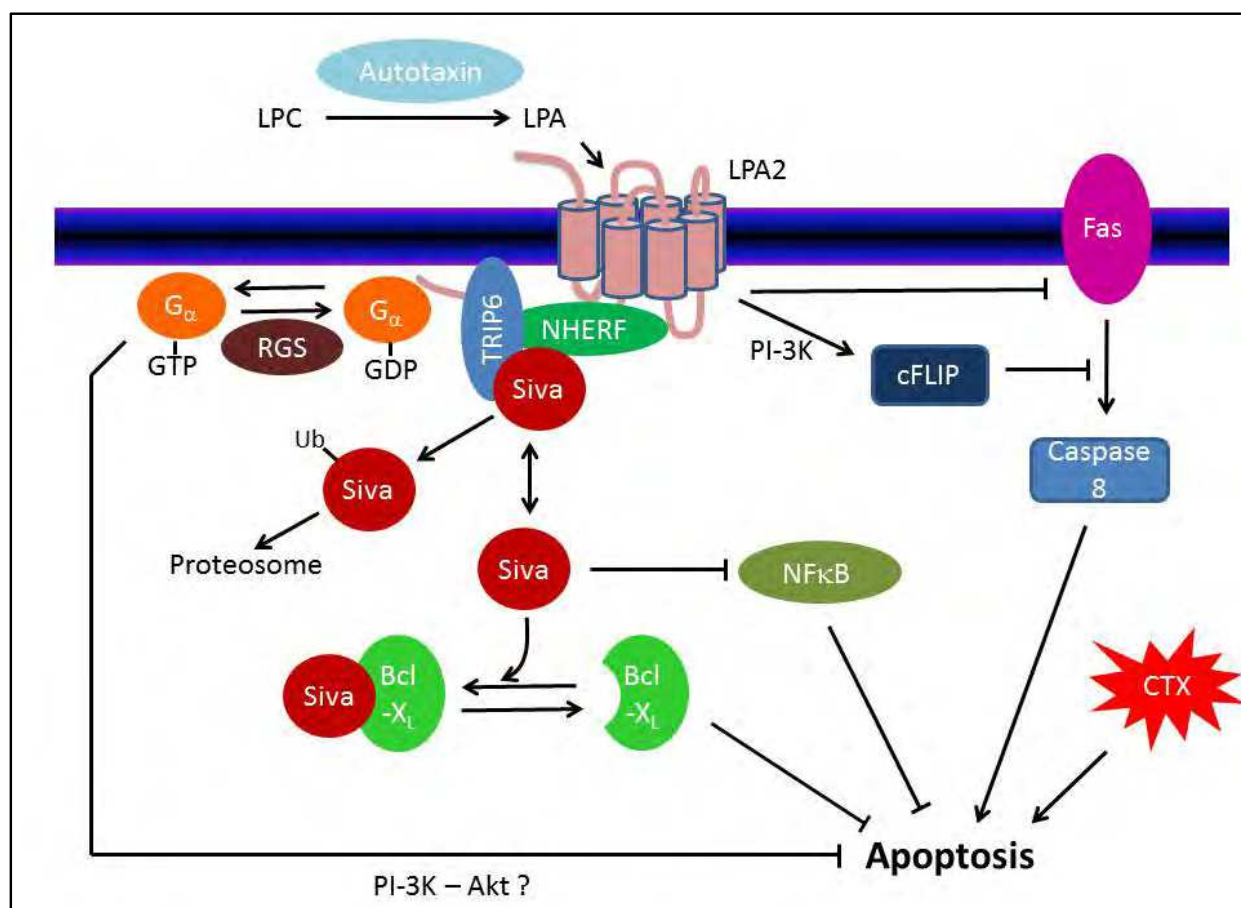


Fig. 2. Regulation of chemoresistance by LPA and autotaxin. CTX denotes chemotherapy, PI-3K, PI 3-kinase.

The observation that RGS and Siva pathways both contribute to chemoresistance in different cell lines highlights the point that there are multiple mechanisms that can cause drug resistance. Thus, if the signalling pathways that are activated by LPA receptors are used as therapeutic targets to restore chemosensitivity, it may be necessary to develop several different therapeutic agents and use them in accordance with the particular pathway that is driving chemoresistance in a individual patient's tumor. If multiple pathways promote resistance, several drugs may be necessary. Alternatively, it may be more straight forwards to develop drugs which either inhibit the LPA receptor(s) or prevent the production of LPA itself.

As well as contributing to resistance to chemotherapy, autotaxin also confer resistance to histone deacetylase inhibitors. HDAC3 and HDAC7 repress the expression of autotaxin. Consequently, exposure to the HDAC inhibitor trichostatin (TSA) increases the expression of autotaxin and the subsequent production of LPA inhibits apoptosis induced by TSA. This suggests that autotaxin confers resistance to HDAC inhibitors (Li et al. 2011). One clinical use of autotaxin inhibitors may be in combination with HDAC inhibitors.

#### 4. Is autotaxin a valid target in ovarian cancer?

A starting point for drug discovery is "target validation" - a process in which data is amassed to give confidence that inhibiting a particular drug target will afford the desired therapeutic outcome. The foregoing discussion highlights several points in the LPA

signalling pathway which might provide drug targets to treat ovarian cancer. Drugs could be developed which: inhibit the synthesis of LPA; increase the catabolism of LPA; up-regulate LPA binding proteins to sequester LPA; inhibit LPA binding to its receptors or inhibit LPA receptor expression; inhibit downstream signalling. (Note that strategies to modulate the tumor environment are already being explored as inhibitors of the VEGF pathway, e.g. bevacizumab, are currently in clinical trials in ovarian cancer and encouraging results have been obtained.)

Although several of these approaches are feasible, in several cases we consider that there is currently insufficient data to identify a drug target as well validated in ovarian cancer. For example, there are multiple signalling pathways activated by LPA receptors. Although experimental data is accumulating, several potential drug targets in these signalling pathways activated by LPA receptors require validation in additional cell lines and evaluation in clinical samples. Until such data is forthcoming, we consider that developing drugs which inhibit the synthesis of LPA or which inhibit LPA receptors are currently the most promising avenues. As we have discussed, there are difficulties with these approaches too. The complexity of the LPA pathway suggests to us that it may be difficult to gather robust target validation data with preclinical studies alone, and that well designed clinical research with inhibitors of autotaxin, iPLA2 or LPA receptors will be necessary to confirm the best approach(s). Thus, for the remainder of this review we will focus on autotaxin as one potential target to inhibiting the LPA pathway.

## 5. Current status of autotaxin inhibitors

To date a number of metal chelators, lipid analogues and non-lipid small molecules have been discovered to be inhibitors of autotaxin. In this section we have concentrated on recent reports of small molecule inhibitors of autotaxin.

Cui and Macdonald have developed a series of tyrosine-derived  $\beta$ -hydroxyphosphonates as analogues of LPA that display activity as inhibitors of autotaxin (Cui et al. 2007; Cui et al. 2008). The synthesis of this series of compounds is highlighted in Figure 3. The sodium borohydride reduction step gave rise to a mixture of two diastereoisomeric products that were separated and isolated by column chromatography. In the initial publications (Cui et al. 2007; Cui et al. 2008) the relative stereochemistry at the new chiral centre had not been determined, but later work from this group on a more advanced series of inhibitors gave insight to the relationship between stereochemistry and activity in the lead compounds (East et al. 2010). From an initial series of targets prepared ( $R^1 = C_{15}H_{31}$ , variation of  $R^2$ ), the most active compound to be identified was compound **1a**, derived from *S*-tyrosine and later confirmed to have the relative stereochemistry shown (Fig. 3), which was able to inhibit 73% of autotaxin activity when tested at a concentration of 1  $\mu$ M. The *syn* isomer, **1b**, was less active achieving 37% inhibition at the same concentration. Interestingly, the corresponding isomers of compound **1** prepared from the enantiomer *R*-tyrosine did *not* show potent inhibition of autotaxin even though they contained the same pyridyl subunit. Structural modification based around varying the length of, or incorporation of unsaturation into, the lipophilic side chains ( $R^1$ , Fig. 3) of compounds **1a** and **1b** did not result in an increase in activity from that originally seen with **1a**.

In a follow-up study (East et al. 2010) the SAR of the pyridyl region was further explored and important structural features were determined to be: the nitrogen heteroatom, the presence of the methoxy substituent, the presence of methyl groups. Extending the alkyl

chain of the alkyloxy substituent to ethyl or propyl led to a fall in activity. Activity was retained on removing the heteroatom as long as the methoxy group and methyl substitution were also retained. In all cases the *anti*-isomer was more active than the *syn* isomer.

Docking studies were carried out on compound **1a** and suggested the proximity of the phosphonate to the two zinc centres at the active site and that the lipophilic side chain was able to fill a large lipophilic pocket, thought to bind the lipid tail of LPC. An aromatic-guanidine binding interaction was also suggested between the benzyl substituent and Arg<sup>456</sup> and a weak H-bonding interaction between the methoxy substituent and Lys<sup>209</sup> within the hydrophilic leaving group pocket of autotaxin. The degree of interaction was dependent upon the electron density of the aryl ring, with more electron rich substituents on the pyridyl ring favouring the interaction. Interestingly the aromatic ring of the tyrosine unit appeared to act solely as an appropriate spacer unit between the more important pharmacophore groups.

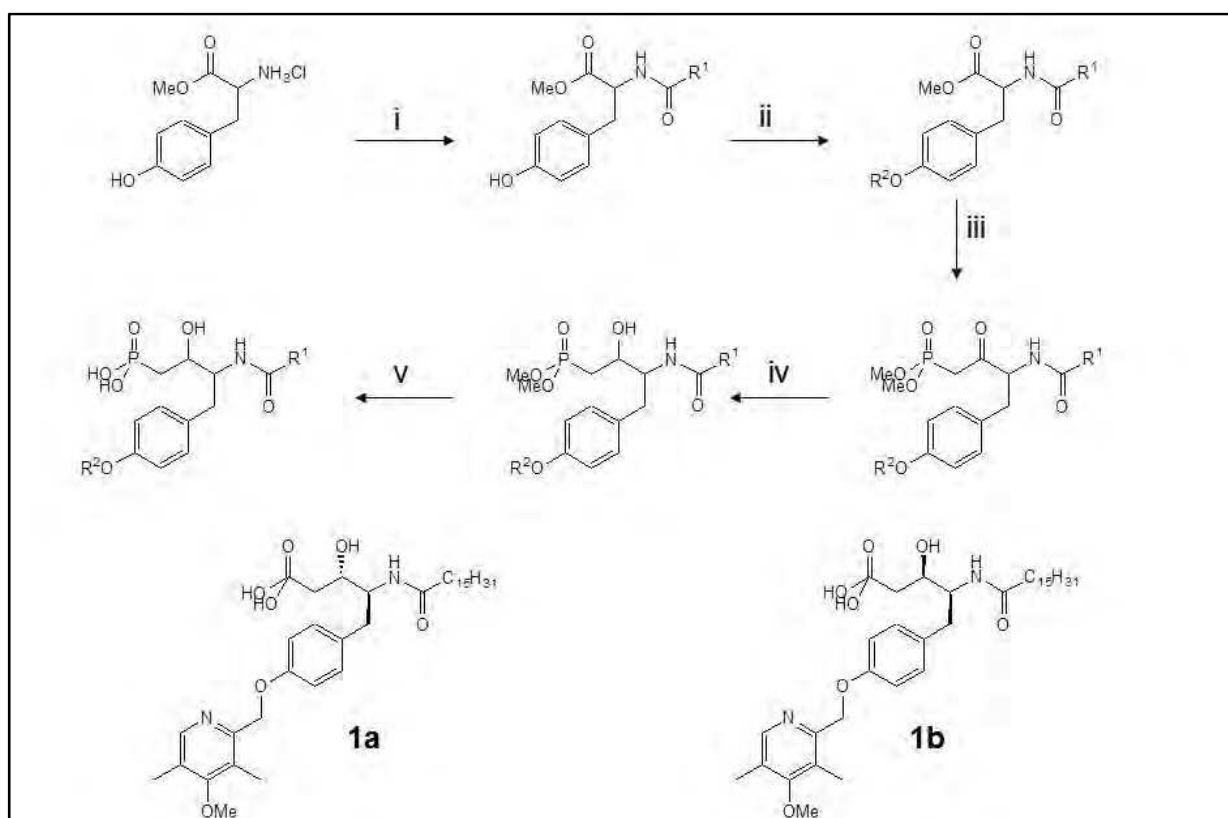


Fig. 3. Tyrosine based inhibitors of autotaxin. (i) Acid chloride, Et<sub>3</sub>N, DCM, 0 °C, 3 h; (ii) Appropriate mesylate, K<sub>2</sub>CO<sub>3</sub>, 18-crown-6, acetone, reflux, overnight; (iii) *n*-BuLi, dimethyl methylphosphonate, -78 °C, 3 h; (iv) NaBH<sub>4</sub>, THF, EtOH, 0 °C, 2 h; (v) TMSBr, pyridine, DCM, rt, 4 h, then H<sub>2</sub>O/MeOH overnight.

A study by Ovaa and co-workers (Albers et al. 2010; Albers et al. 2010) on a collection of *ca.* 40,000 compounds has allowed the identification of a group of thiazolidinediones as autotaxin inhibitors. The general class of compound was prepared as outlined in Figure 4. From the initial screen, compound **2** was found to be the most active (IC<sub>50</sub> = 56nM) and was selected for further optimization. Although structural variation at the benzylidene and benzyl groups did not lead to an increase in activity, the opportunity was taken to investigate pharmacophoric variation of the carboxylic acid substituent.

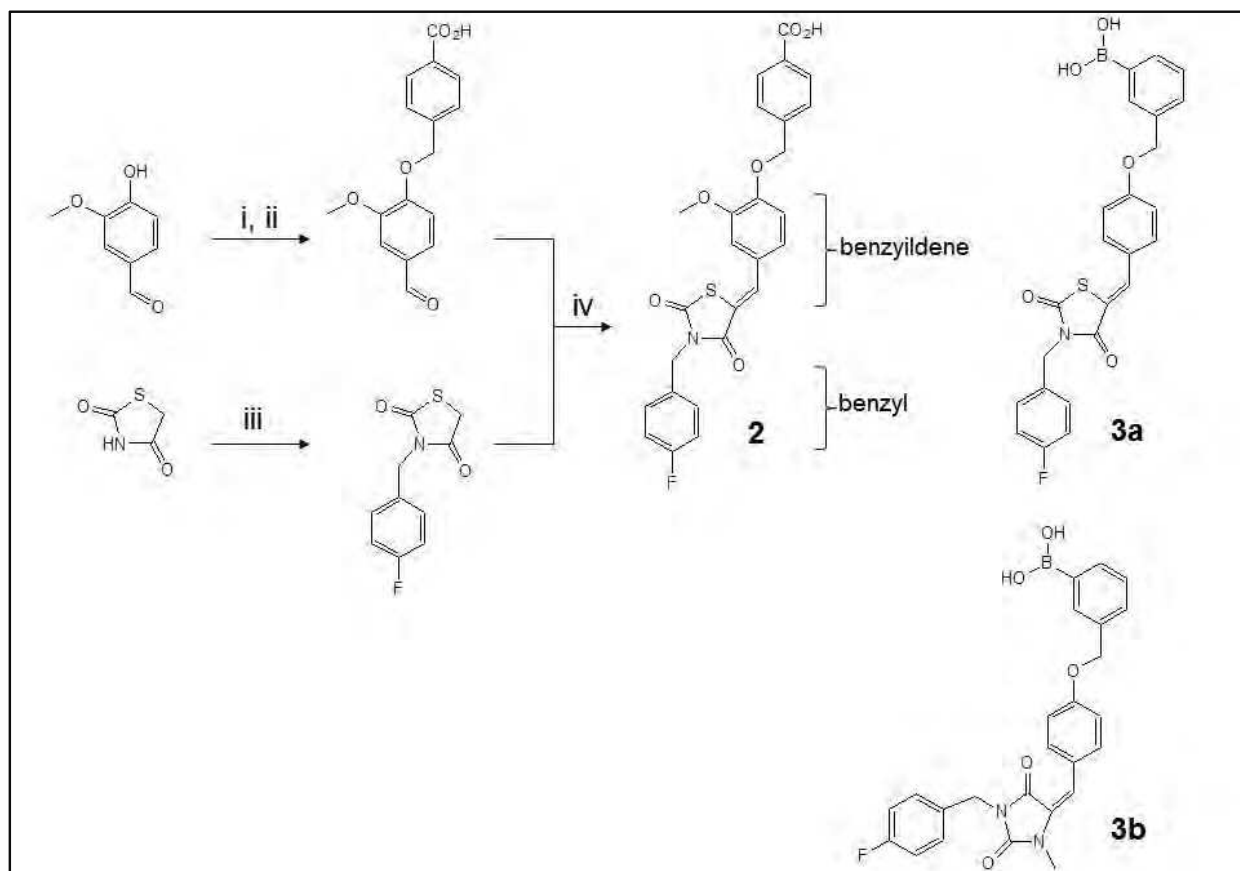


Fig. 4. (i) KOH, DMSO, appropriate benzyl bromide (as methyl ester), rt, 30 min; (ii) NaOH, DMSO/H<sub>2</sub>O, reflux, 4 h, 91% yield; (iii) NaH, DMF, rt, appropriate benzyl chloride, 22 h, 74% yield; (iv) piperidine, EtOH, reflux, 20 h, 63% yield.

Replacing this group with a boronic acid moiety gave compound **3** that was found to be a potent inhibitor of autotaxin both *in vitro* and *in vivo* (IC<sub>50</sub> = 6 nM). These results were rationalized on the basis that since the carboxylic acid group in **2** was expected to bind close to the active site threonine (Thr<sup>210</sup>) in autotaxin, a boronic acid moiety might be expected to do the same. There was a precedent for this since the proteasome inhibitor bortezomib binds to a threonine oxygen nucleophile at the active site through a boronic acid group (Groll et al. 2006). The boronic acid-based thiazolidinediones showed greater affinity for autotaxin and are expected to show improved selectivity over other hydrolytic enzymes. The boronic acids such as **3** are expected to have the same binding site as the original lead **2**, but they show mixed-type inhibition rather than the competitive inhibition displayed by **2**. Ovaa has recently extended this work and has reported that the imidazolidine analogues such as **3b** show a similar level of activity to **3a** (Albers et al. 2011).

Virtual screening techniques have been used by Parrill, Baker and co-workers (Parrill et al. 2008; Hoeglund et al. 2010; Hoeglund et al. 2010; North et al. 2010). This has led to series of autotaxin inhibitors with pipemidic acid or phthalimide cores and related compounds (fig. 5). Of the pipemidic acid-based inhibitors (eg **4**, **5**, fig. 5), compound **4** (IC<sub>50</sub> = 1.6 μM) was used as a lead to investigate the activity of a range of analogues with varying substitution on the pendent benzene ring. The synthetic approach was straightforward (fig. 6), starting with commercially available pipemidic acid and a range of substituted phenyl isothiocyanates to produce 30 compounds for evaluation. Themes to emerge were that *meta*

substitution is preferred regardless of substituent, suggesting steric or conformational preferences rather than electronic effects are playing a role here. Within the *meta* class of compounds, inhibition was improved in the order: OMe<F<Cl<I,<CF<sub>3</sub>, reflecting neither size nor electronic trends. A single *meta*-trifluoromethyl group was preferred over two, and the singly substituted compound showed three times greater affinity for the enzyme than the original lead compound **4**. Of the compounds screened, compound **7** emerged as the most potent analogue in this study (IC<sub>50</sub> = 0.9 μM; K<sub>i</sub> = 0.7 μM), and showed competitive inhibition.

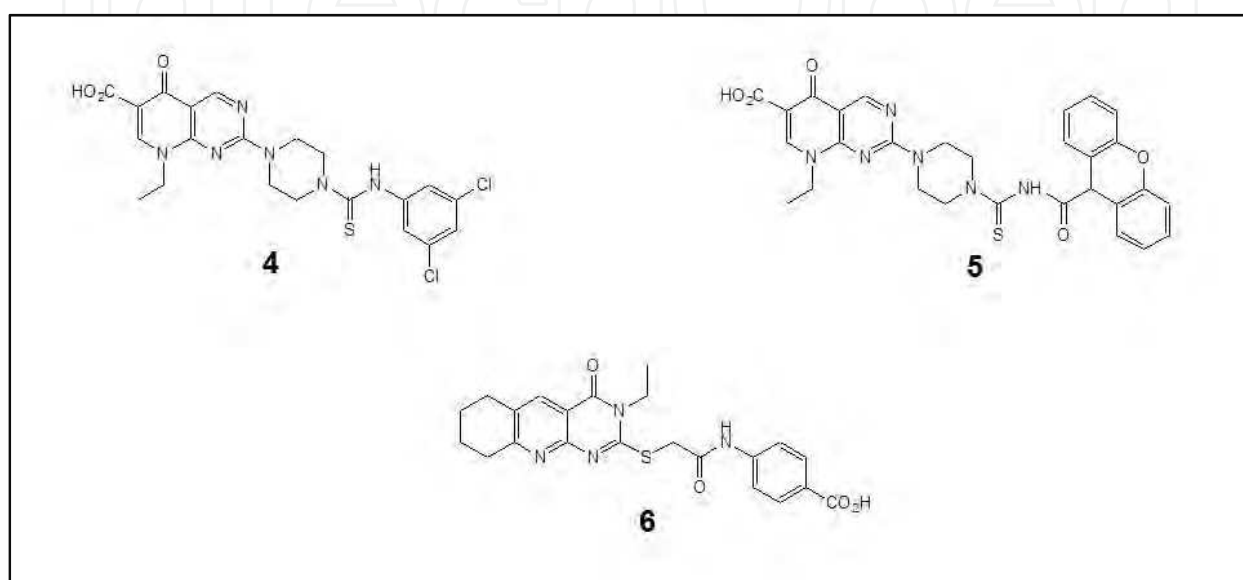


Fig. 5. Hits identified by virtual screening by Parrill and co-workers all of which inhibited autotaxin with IC<sub>50</sub> ~ 2μM. Compounds **4** and **5** are piperidic acid derivatives.

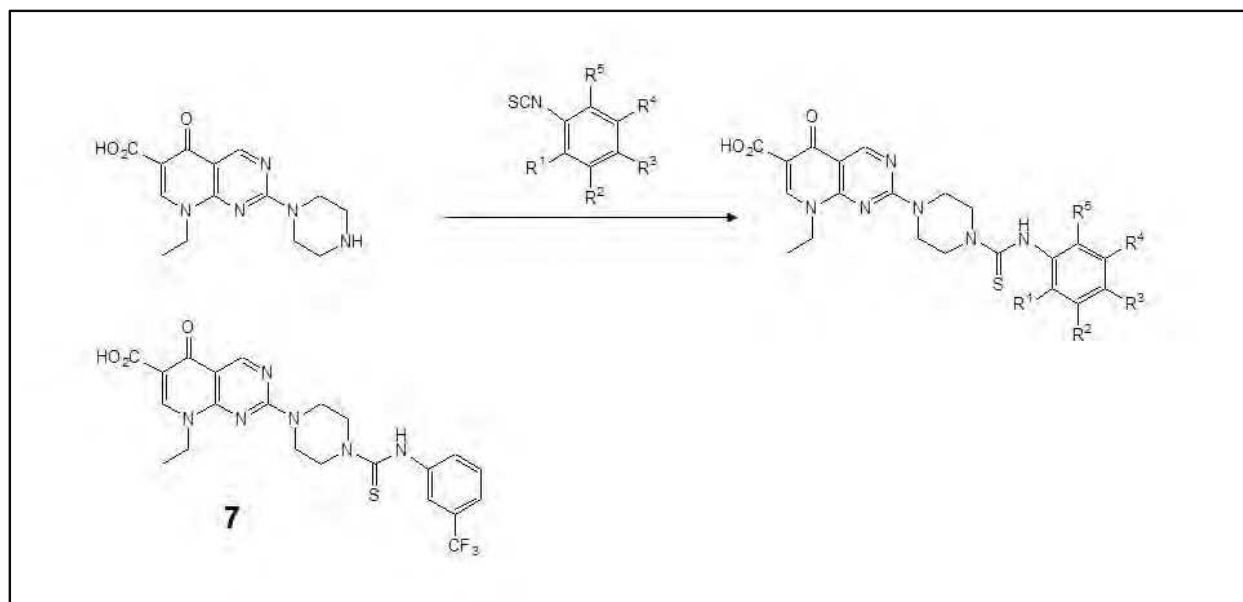


Fig. 6. Synthesis of autotaxin inhibitors based on piperidic acid. Compound **7** was the most potent analog reported (IC<sub>50</sub> ~ 1 μM).

The phthalimide-derived small molecule lead inhibitors that were identified in the virtual screen by the Parrill and co-workers were also further evaluated including some dimeric examples, (Fig. 7) (Hoeglund et al. 2010). It is noticeable that these compounds contain terminal functionality that would be expected to contribute towards binding to zinc at the active site. Compounds **8** and **10** showed mixed-mode inhibition, whereas compound **9** showed competitive inhibition of autotaxin.

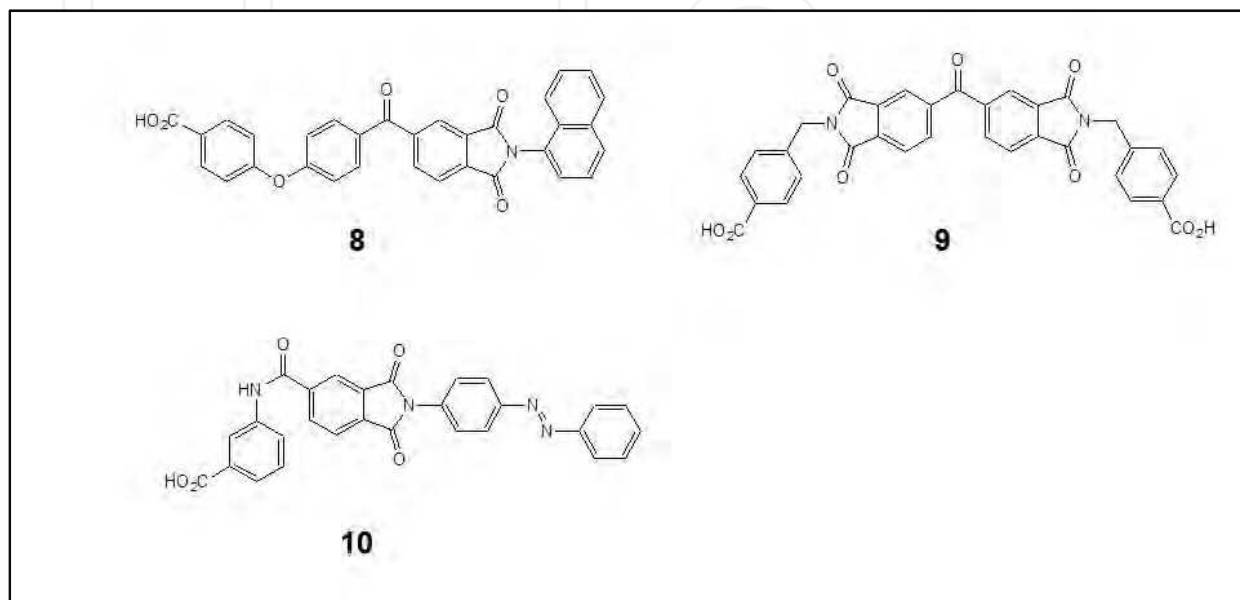


Fig. 7. Phthalimide-derived autotaxin inhibitors, all of which showed moderate potency ( $IC_{50} = 5 - 10\mu M$ ) against autotaxin.

A recent report by Miller and Tigyi (Gupte et al. 2011) has built upon work carried out by Ferry and co-workers reporting that compound **11** (known as S32826, fig. 8; (Ferry et al. 2008)) possessed nanomolar activity as an autotaxin inhibitor. S32826 was inactive when evaluated using *in vivo* systems, and it has been presumed by Miller and Tigyi (Gupte et al. 2011) that this is due to the propensity for hydrolysis of the amide bond, making S32826 relatively unstable. In their own work Miller and Tigyi report a series of benzyl and naphthalene methyl phosphonic acid-derived compounds, of which **12** and **13** are most active, as inhibitors of autotaxin and that possess anti-invasive and anti-metastatic activity (Gupte et al. 2011). The synthetic approach to compound **12** (fig. 8) begins with a Heck coupling of the benzoic acid derivative to introduce the long alkyl side chain.

Compound **12** shows 94.8% inhibition of autotaxin and has an  $IC_{50}$  of  $0.17\ \mu M$ , with a  $K_i$  of  $0.27\ \mu M$  and displays a mixed mode of inhibition. In addition to inhibiting the invasion of MM1 hepatoma cells *in vitro* in a dose-dependent fashion, compound **13** significantly decreases lung metastasis of B16-F10 syngeneic mouse melanoma. Compound **12** has an average terminal half-life of  $10 \pm 5$  hours and causes a long-lasting decrease in plasma LPA levels.

Prestwich has recently reported the synthesis of a hydroxylated S32826 analogue, (fig. 8, **14**), that retains acceptable levels of solubility ( $4\text{mg/ml}$ ) whilst maintaining its potency as an inhibitor of autotaxin ( $K_i = 24.2\ \text{nM}$ ) and has potential for *in vivo* utility (Jiang et al. 2011). Compound **14** is currently undergoing further preclinical study.

Metabolically stable analogues of LPA, **15** and **16**, were designed by Prestwich and co-workers (Jiang et al. 2007). Compound **15** showed selective agonist activity for LPA<sub>2</sub>, whereas **16** is a selective antagonist of LPA<sub>4</sub> and indeed is the first antagonist of this receptor to be reported. Compound **15** was found to be as effective as natural LPA as an inhibitor of autotaxin. Arguably the most interesting compound though, **16**, showed pan-antagonism of LPA GPCR's and was also active as an autotaxin inhibitor, thus having potential in anticancer and anti-metastasis models in cancer therapy (Zhang et al. 2009).

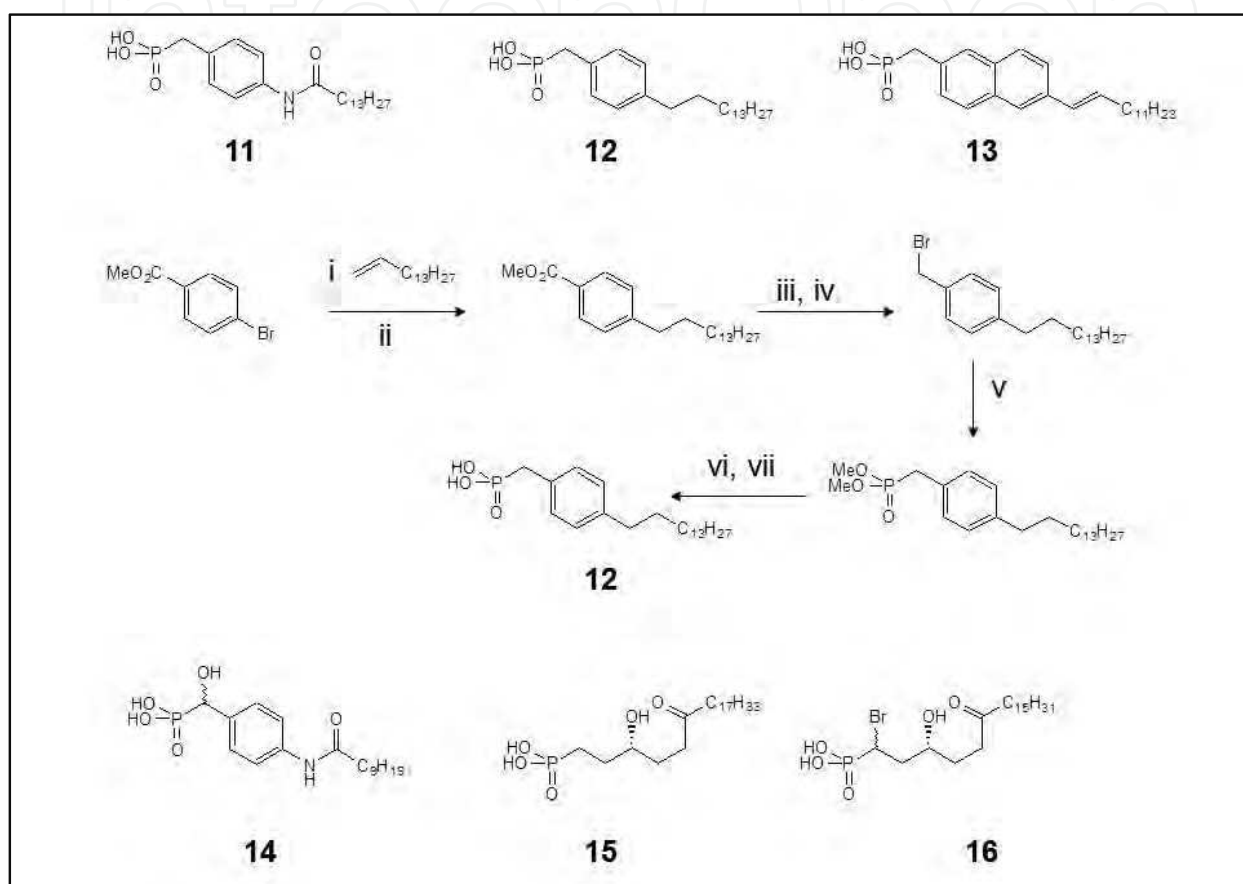


Fig. 8. (i) Pd(OAc)<sub>2</sub>, Et<sub>3</sub>N, DMF, reflux, 16 h; (ii) LAH, THF, 0 °C-rt, 4 h; (iii) H<sub>2</sub>, Pd/C, MeOH, rt, 2 h; (iv) PBr<sub>3</sub>, Et<sub>2</sub>O, rt, 30 min; (v) P(OMe)<sub>3</sub>, reflux, 18 h; (vi) TMSBr, CH<sub>3</sub>CN, reflux, 1 h; (vii) MeOH, rt, 30 min.

## 6. Structure of autotaxin and molecular modelling to aid drug design

### 6.1 Autotaxin structure

The availability of data describing the structure of autotaxin is a superb tool to support drug discovery. Of the 8 protein crystal structures of autotaxin (*ENPP2*) that have thus far been described (Hausmann et al. 2011; Nishimasu et al. 2011) only one (2XRG.pdb; Table 1) has a small drug-like molecule bound in the active site. There are two unliganded structures. The others contain a range of phospholipids and LPA analogues with a variety of fatty chains and LPA analogues. The overall architecture of autotaxin is shown in figure 9. The N-terminus begins with a pair of somatomedin-B like (SMB) domains (residues 56-96 and 96-140) which lead into a phosphodiesterase domain (160-539) which contains a catalytic zinc binding site, a

lasso loop (residues 539-590) and finally a nuclease domain (residues 539-862). It has also been suggested that a glycan chain located between the phosphodiesterase and nuclease domains is essential for correct folding of the protein. Strong bonding and electrostatic interactions are observed between the phosphodiesterase and the C-terminal region of the NUC domains including a disulphide cysteine bridge between residues 413 and 805 along with seven hydrogen bonds and nine salt bridges. This, combined with the extension of the Lasso loop from the PDE domain around the NUC domain, makes for a tight and well-ordered protein.

PDB CODE	Description	Reference
2XRG.pdb	Rat ATX with ligand	Hausmann et al
2XRN.pdb	Rat ATX no ligand	Hausmann et al
3NKM.pdb	Mouse ATX	Nishimasu et al
3NKN.pdb	Mouse ATX 14:0 LPA	Nishimasu et al
3NKO.pdb	Mouse ATX 16:0 LPA	Nishimasu et al
3NKP.pdb	Mouse ATX 18:1 LPA	Nishimasu et al
3NKQ.pdb	Mouse ATX 18:3 LPA	Nishimasu et al
3NKR.pdb	Mouse ATX 22:6 LPA	Nishimasu et al

Table 1. Structures of human autotaxin available in the Protein database. (www.pdb.org)

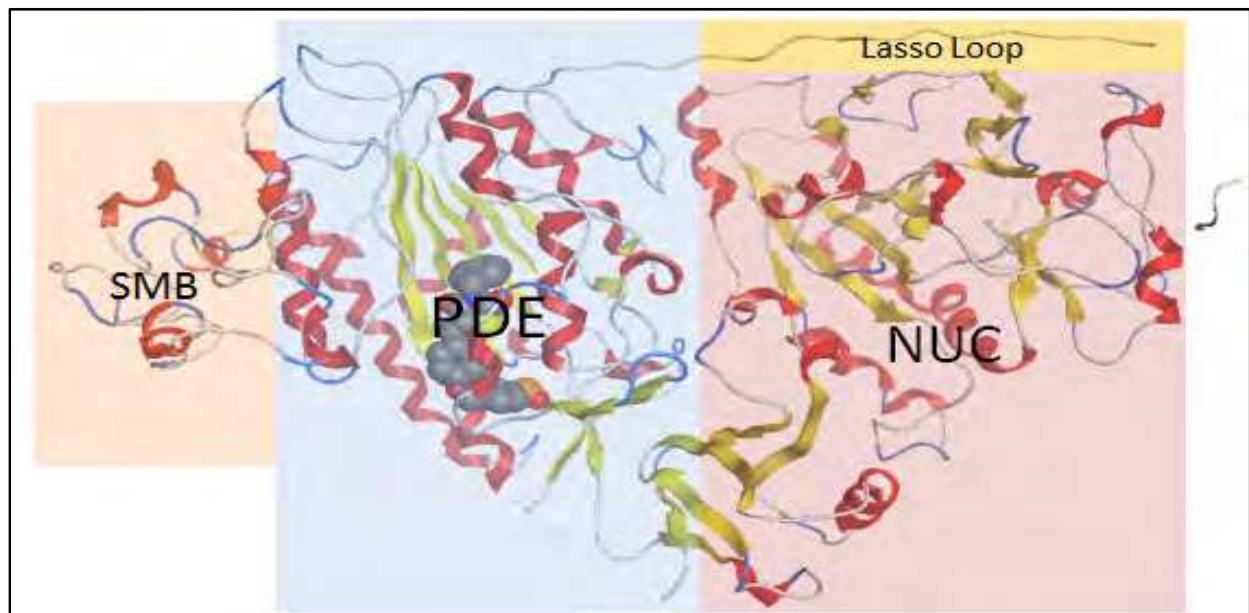


Fig. 9. The domain structure of autotaxin. The figure shows the location of the somatomedin domain (SMB), the phosphodiesterase domain (PDE) containing the catalytic site, the lasso loop and the nuclease domain (NUC).

The SMB domains are required for integrin binding and so may play a role in recruiting autotaxin to the cell surface where LPA receptors are located. A tunnel between one of the SMB domains and the ligand binding pocket has been suggested to facilitate delivery of LPA to cell surface receptors (Tabchy et al. 2011). The location of the tunnel compared to the catalytic site is shown in figure 10. Many lipophilic molecules are transported bound to protein carrier, such as albumin, so it seems reasonable that the tunnel fulfils a carrier role.



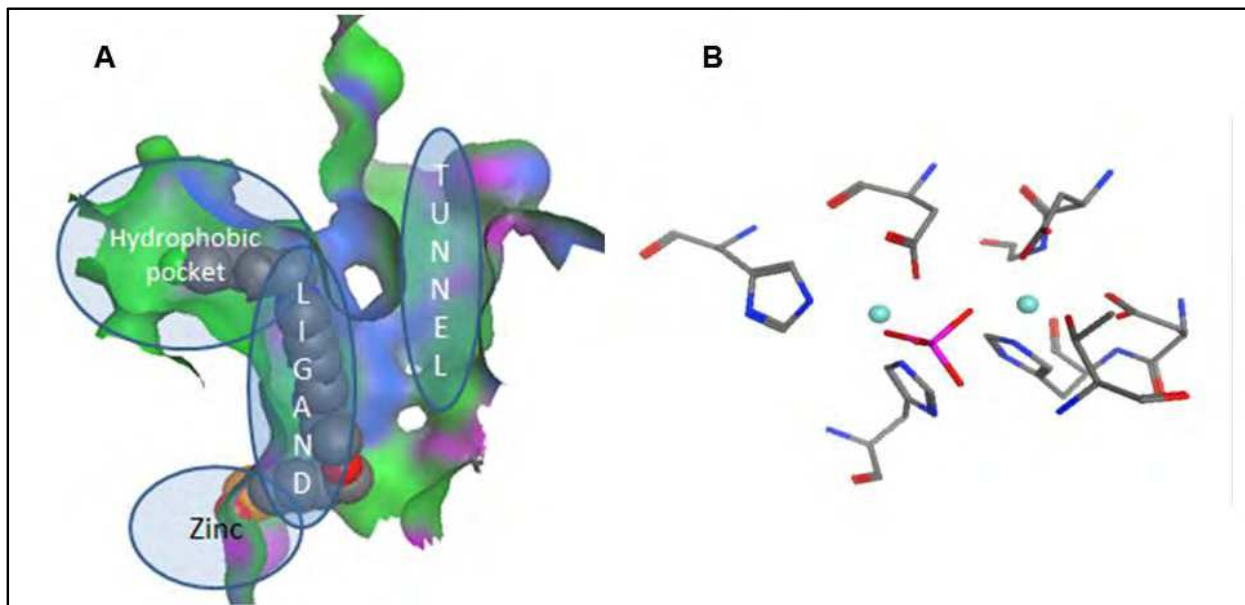


Fig. 10. **A.** A cut away diagram of the hydrophobic channel (right) adjacent to the ligand binding pocket. The main features of the binding site are highlighted with ovals. **B.** A close-up view of the local environment of catalytic zincs. The residues are coloured: grey, carbon; blue, nitrogen; red oxygen, light blue, zinc.

## 6.2 Ligand binding site

The overlay of several published X-ray structures of autotaxin shows a high conservation of ligand binding sites with only a few mobile residues. It is important to consider these as they may change the shape of the pocket or the electronic environment presented to the ligand and consequently affect ligand binding (fig 11).

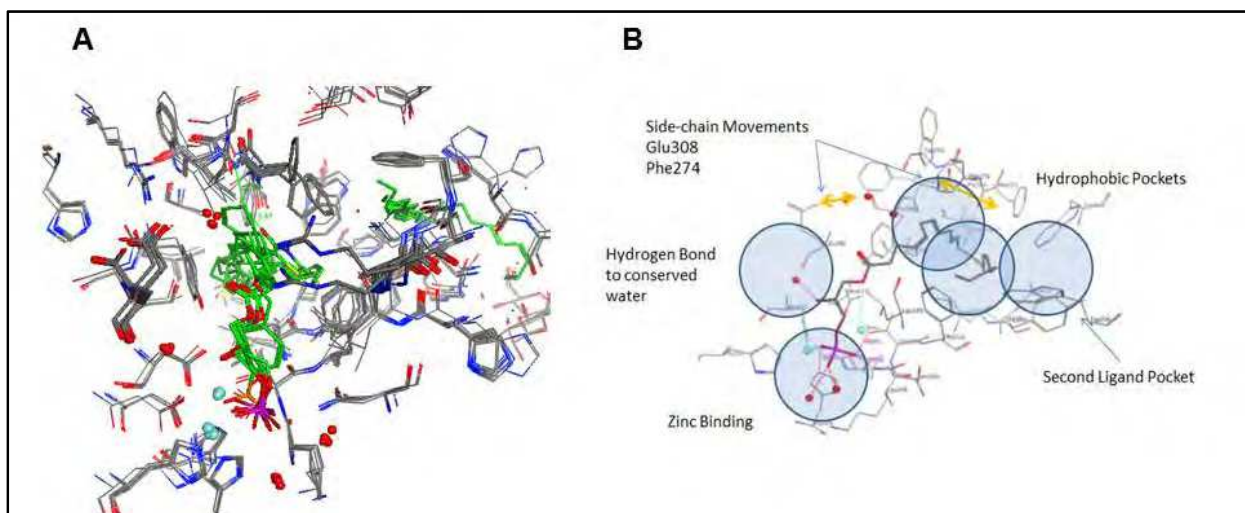


Fig. 11. **A.** The overlay of the active sites of 8 published protein structures of autotaxin. Residues whose positions have moved significantly between the structures are shown emboldened. Carbon atoms in LPA are shown in green whereas autotaxin carbon atoms are coloured grey; other atoms are coloured purple (phosphate), blue (nitrogen), red (oxygen) and light blue (zinc). **B.** An annotated view of the main features of the ligand binding site

The main features of the catalytic site are shown in figure 11. There are two di-cationic zinc atoms (fig 10 and fig. 11). The first is fully co-ordinated with a histidine, and threonine and two aspartate residues. The second zinc ion is co-ordinated by an aspartate and two histidine residues, leaving it available for co-ordination by a further negatively charged species such as phosphate or carboxylate.

Asn<sup>231</sup> and Thr<sup>210</sup> have been shown to be crucial for catalysis. These are located close to one zinc ions and provide a hydrogen bonding environment. Asn<sup>231</sup> gives a preference for hydrogen bond acceptors while Thr<sup>210</sup> provides an opportunity for irreversible (covalently bonded) ligands such as boronic acids to react. The conserved water that is held by hydrogen bonds between Asp<sup>311</sup> and Glu<sup>308</sup> can also play a role in control of ligand orientation and enantio-selectivity of ligand recognition.

The remainder of the site consists of a large hydrophobic pocket which has recognition features for the lipophilic chain of LPA. Within this pocket most of the side chains appear to be less mobile, although there are however three notable exceptions, Glu<sup>308</sup>, Phe<sup>274</sup> and Arg<sup>244</sup>, some of which may help determine the potency with which ligands bind to autotaxin. Movement of Glu<sup>308</sup> seems to displace a water molecule. Phe<sup>274</sup> is mobile and unresolved in some structures but is shown to occupy various positions (fig. 12) in the hydrophobic pocket in others crystal structures in some cases moving to accommodate the ligand. We speculate that movement of this residue allows LPA to move from the active site into the hydrophobic tunnel. Arg<sup>244</sup> is also mobile but its location adjacent to the solvent suggests that it may not be as important for ligand recognition.

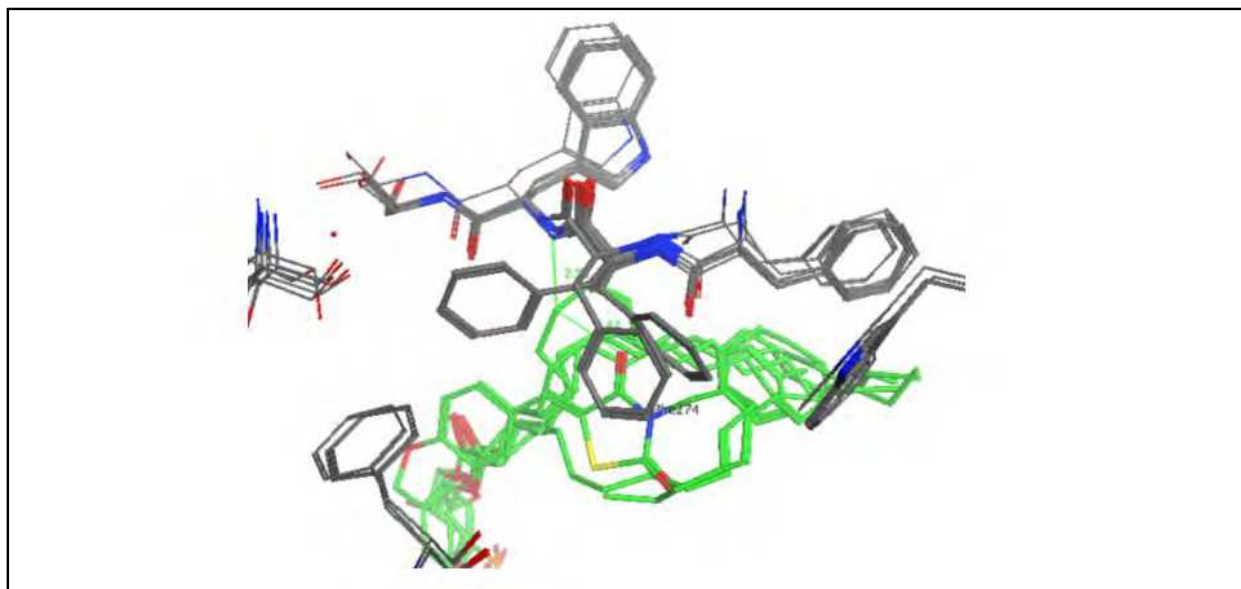


Fig. 12. This diagram shows the movement of side-chain Phe<sup>274</sup> (grey, in centre of diagram) within the ligand binding site. The green shows the bound boronic acid inhibitor.

### 6.3 Docking methods

We have used the published crystal structures in an effort to understand the activity of several of the compounds described in section 5. In particular we evaluated whether the reported ligands were involved in zinc binding or whether they could occupy the hydrophobic pocket. Pharmacophoric overlay of autotaxin ligands has previously been reported to give a reasonable explanation of their relative binding modes by assuming that

the ligands all make the same interactions within the autotaxin pocket (North et al. 2010). Here, however, we have used docking models of the catalytic zinc pocket to identify and compare the interactions of reported inhibitors. Ligands were placed within the binding pocket by aligning them with the pharmacophore map that had been overlaid onto the inhibitor bound in structure 2XRG. The steric requirements of the pocket were then used to refine our understanding of the activities of the ligands.

The pharmacophore map of the active site was constructed based on the binding of the boronic acid inhibitor HA155 (fig. 13). Docking to this map was performed using MOE software from the Chemical Computing Group. The placements of ligands were constrained to pharmacophoric points within the active site and refinement was performed with the MMFF94x force field (Halgren 1996).

The docking model faithfully reproduced the position of the ligand from 2xrg.pdb and its close analogues. This gives us confidence in the docking models used. While the pharmacophore map suggests a rich hydrogen bonding network around the ligand, most of the “linker” region of the ligands fail to interact with the pocket other than with a few hydrophobic contacts. The conserved nature of the ligands’ hydrogen bonding motif, as can be seen from previous pharmacophore modelling efforts (North et al. 2010), suggests that there may be unresolved water atoms which are located in the site (large blue spheres in fig. 13).

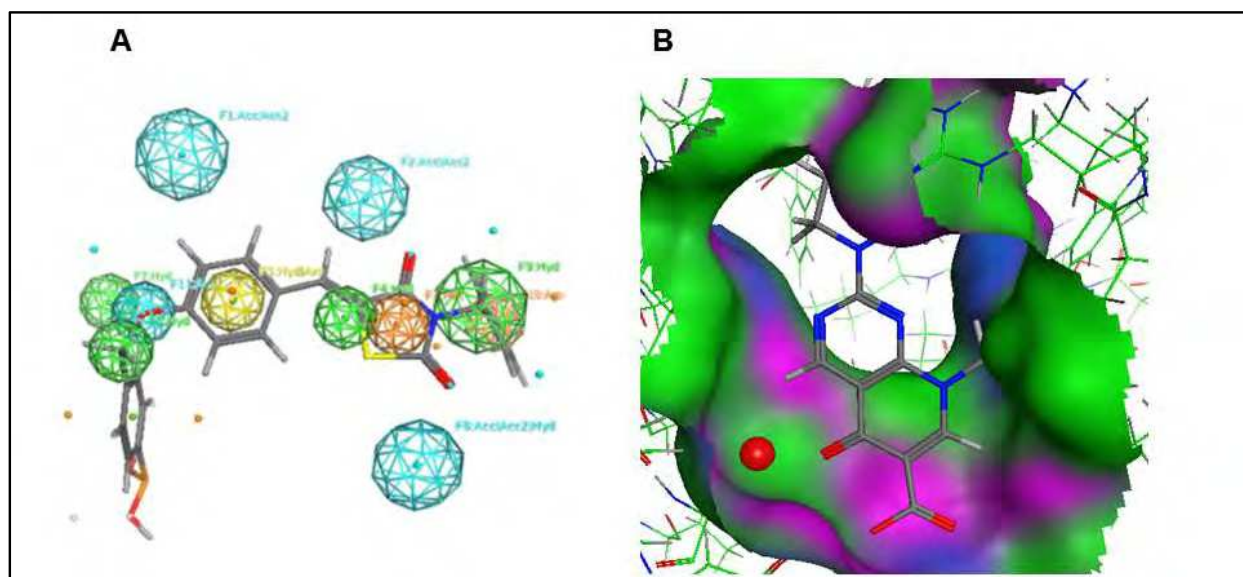


Fig. 13. **A** Pharmacophoric map and shape of ligand from 2XRG.pdb which takes into account the steric and electronic requirements of the pocket when placing the ligand. (Blue Mesh= hydrogen bond acceptor projected points, Green Mesh=Hydrophobic areas, Brown/yellow Mesh=Aromatic centers) **B**. The zinc binding portion of molecule 5 after docking to the pharmacophore map in the binding site.

The best fit to the zinc pocket was obtained for the carboxylic acid group from molecules 4 and 5 which appear to satisfy all the steric and electronic requirements of the local pocket (Fig below) When the hydrophobic tail of the docked molecules is prevented from occupying the ligand binding pocket because of a lack of ligand flexibility, it is often placed by the docking software in the tunnel region of the protein. This may be influenced by Phe<sup>274</sup> which may act as a switch closing one or the other of these sites. Stabilization of this residue may contribute to ligand affinity.

Ligands which bind with high affinity interacted with both the zinc ion and also occupied the hydrophobic pocket e.g. with a benzyl group. In contrast, ligands which bind with low affinity failed to bridge the zinc ion and the hydrophobic portion of the ligand binding site. This leads us to conclude that for ligands to bind with high affinity, they should preferably bind both these sites. Docking studies also suggest that the hydrophobic tunnel provides an alternative location for ligands but these ligands identified so far fail to achieve better than micromolar activities. These observations may be used to design improved autotaxin inhibitors.

## 7. Potential clinical uses of autotaxin inhibitors

Once inhibitors of autotaxin complete preclinical evaluation, how could these be evaluated in clinical trials? As we have discussed above, autotaxin may not be the only enzyme that contributes to the formation of LPA in ovarian cancer and so it is possible that inhibition of autotaxin may not elicit the desired therapeutic effect. It will be important, therefore, to include in early clinical trials a measurement of the change in LPA in ascites following treatment with an autotaxin inhibitor and to establish biomarkers (e.g. measurement of autotaxin in ascites fluid) to stratify the patients which are likely to respond.

The data we have reviewed also suggests a number of different settings in which autotaxin inhibitors could be used. Autotaxin inhibitors may be useful to inhibit the growth of primary tumors or to inhibit tumor cell migration, invasion and metastases. Clinical trials to evaluate these may differ somewhat, for example using different surrogate endpoints (tumor shrinkage versus decreased metastasis). Different schedules of drug administration may also be appropriate. To cause tumor cell death, relatively short term treatment with the drug may suffice, but suppression of metastasis may require prolonged treatment. This highlights the importance of considering the therapeutic goal that is being evaluated with an autotaxin inhibitor at the outset.

As we have discussed, there is a large body of evidence indicating a role for LPA in cancer cell migration and invasion. In addition, several studies demonstrate in animal models of metastasis that inhibitors of autotaxin reduce colonization of the lung by tumors cells (Baker et al. 2006; Gupte et al. 2010; Gupte et al. 2011). Thus, one potential clinical use of autotaxin inhibitors is to inhibit metastasis. Unfortunately, many ovarian cancer patients present with advanced disease, and significant dissemination of the tumor within the peritoneal cavity has already taken place by the time of diagnosis. Although it may be beneficial to prevent further metastasis and progression to later stage disease, there may also be micro-metastases that are not evident on examination. It is not clear, then, whether inhibiting further metastasis would be helpful. Evaluation of autotaxin inhibitors as anti-metastatic agents in patients with early stage disease will also be challenging. Relatively few patients are diagnosed with early stage disease and these patients generally have a good prognosis, with 90% of patients surviving more than 5 years. A large cohort of patients may also be required to ensure sufficient patients are evaluated who lack pre-existing micro-metastases but who will progress to more advanced disease. The cost of such a large and long trial may be prohibitive unless an alternative is found.

We have also reviewed the substantial evidence linking LPA and autotaxin to cell survival. Autotaxin inhibitors may have an indirect cytotoxic effect or inhibit the growth of primary (and secondary) tumors. This might reflect deprivation of LPA directly causing apoptosis of the tumor cells, or it might reflect a less supportive

microenvironment. In support of this approach, BrP-LPA (Bromophosphonolysophosphatidic acid; **16**, fig. 8) causes regression of breast tumor cells both in 3D *in vitro* models (Xu and Prestwich 2010) and as a xenograft (Zhang et al. 2009), although as the authors clearly state, this drug is also a pan LPA receptor antagonist and its activity cannot be ascribed to inhibition of autotaxin alone. Also, over-expression of autotaxin is sufficient to induce breast cancer, suggesting that selective inhibition of autotaxin may be sufficient to cause regression of comparable tumor types. An alternative to using an autotaxin inhibitor as a single agent is to use it in combination with chemotherapy and this is supported by the data implicating autotaxin and LPA in chemoresistance that we have discussed. Clearly this strategy has potential in patients who have developed chemoresistant disease, but it may also be useful to increase the response in patients whose tumors are sensitive to chemotherapy.

A pragmatic solution may be to evaluate autotaxin inhibitors first for their ability to inhibit tumor growth (either as a single agent or in combination with chemotherapy) and if this is successful evaluate their use as anti-metastatic agents after drug receives marketing approval. This may mitigate some of the risk associated with following a purely anti-metastatic approach to drug development.

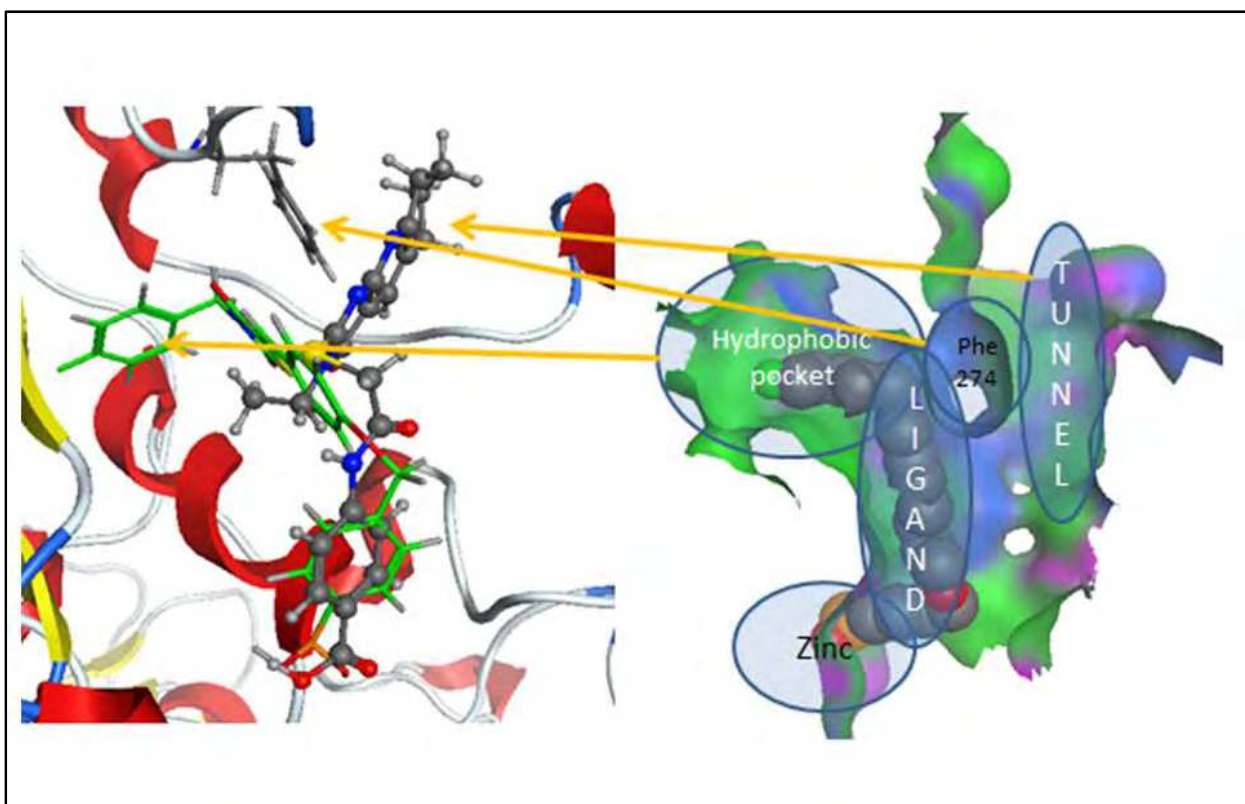


Fig. 14. The hydrophobic “tail” in ligands may occupy the hydrophobic pocket or the hydrophobic tunnel and Phe<sup>274</sup> divides the two hydrophobic ligand binding sites.

## 8. Conclusion

Our understanding of autotaxin, in terms of its biological function, its structure and its potential as a drug target in ovarian cancer is rapidly evolving. Several compounds are

currently undergoing preclinical discovery, and it cannot be long before the first of these enter clinic trials. In this review, we have focused on the role of autotaxin in ovarian cancer, but it also plays a role in other cancer types as well as other pathophysiological conditions such as neuropathic pain. It seems plausible that autotaxin inhibitors will serve as new medicines and perhaps none of these applications is as exciting as the potential to treat drug-resistant ovarian cancer, a disease for which therapeutic options are currently limited.

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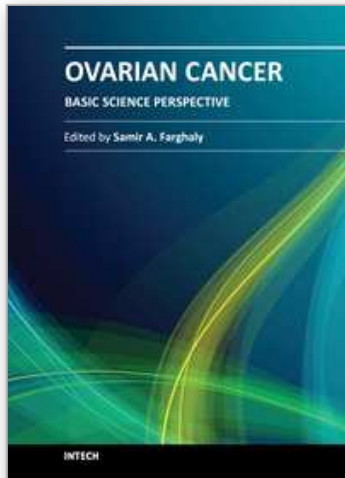
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Worldwide, Ovarian carcinoma continues to be responsible for more deaths than all other gynecologic malignancies combined. International leaders in the field address the critical biologic and basic science issues relevant to the disease. The book details the molecular biological aspects of ovarian cancer. It provides molecular biology techniques of understanding this cancer. The techniques are designed to determine tumor genetics, expression, and protein function, and to elucidate the genetic mechanisms by which gene and immunotherapies may be perfected. It provides an analysis of current research into aspects of malignant transformation, growth control, and metastasis. A comprehensive spectrum of topics is covered providing up to date information on scientific discoveries and management considerations.

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