Chapter 12

Autotaxin – An Enzymatic Augmenter of Malignant Progression Linked to Inflammation

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Additional information is available at the end of the chapter

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1. Introduction

Malignant melanoma cells are incredibly hardy, stemming from their intrinsic defensive nature. These cells inherit unique characteristics, which allowed their non-malignant predecessors, melanocytes, to survive solar ultraviolet radiation and simultaneously provide protection to neighboring cells. Most other cell types would die after such harsh exposure. Unsurprisingly, melanoma cells, which survive ultraviolet radiation, are intrinsically resistant to most chemotherapy.

Although there are numerous molecular reasons for this reality, herein we focus on the causal role of autotaxin (ATX) in melanoma. ATX is a 125 kDa glycoprotein enzyme that was initially discovered in the serum-free medium of A2058 human melanoma cells by Stracke et al. in 1992 [1] (Figure 1). Today, we know much more about this glycoprotein enzyme and how it affects melanoma. Indeed, ATX is highly overexpressed among primary melanomas and metastatic melanomas, in comparison to melanoma *in situ* and in normal skin [2].

In fact, "autotaxin" derived its name based on its initial property as an "autocrine motility factor". The rationale is that A2058 melanoma cells secrete ATX into culture medium and then respond to it with self-stimulated random and directed motility. Even though the amount of ATX in conditioned medium is less than 0.005% of total protein, only miniscule amounts, detected in picomolar and nanomolar concentrations, are needed to promote motility. [1] This suggests a large role for a low-abundant secreted enzyme.

There are five alternatively-spliced isoforms of ATX that are catalytically active [3,4]. The original ATX protein described in 1992 is termed ATXα, whereas the most abundant isoform is ATXβ and is the same isoform responsible for plasma lysoPLD activity [5]. Full length ATX

is synthesized as a pre-proenzyme and is secreted by the classical secretory pathway [6,7]. Secreted ATX binds to cell surface integrin or heparan sulfates through its somatomedin-B-like (SMB) domain. This surface binding is believed to localize LPA production adjacent to LPA receptors [8-12].

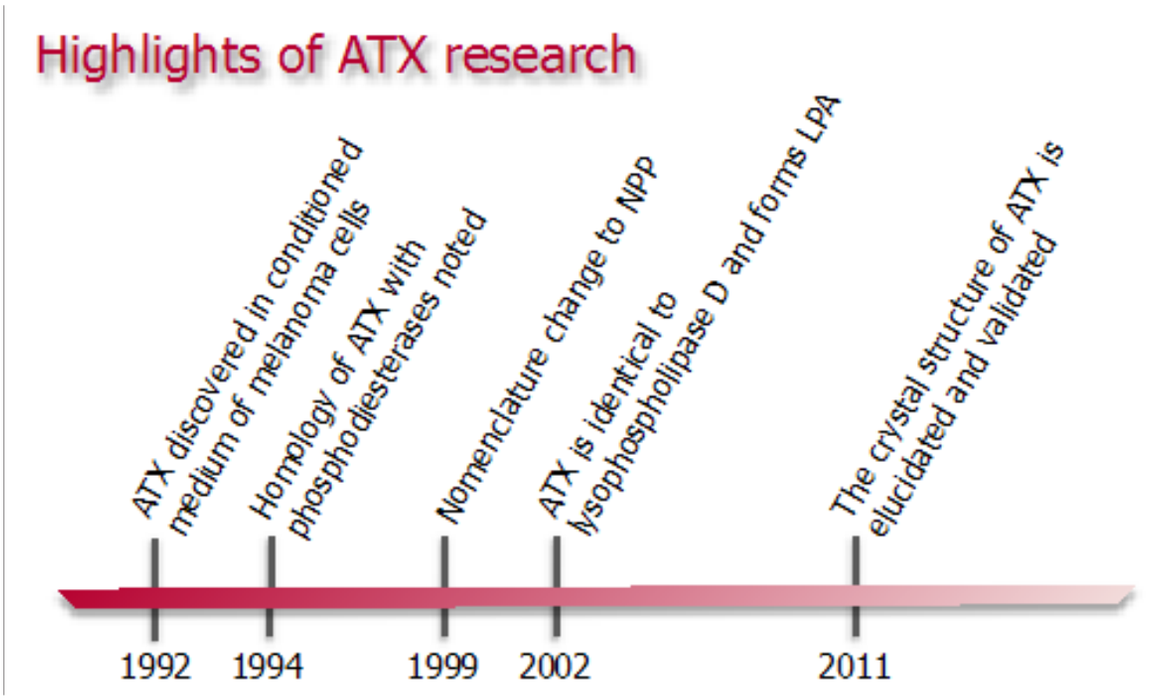

Figure 1. Major milestones in autotaxin (ATX) research. After the discovery of ATX in the conditioned medium of melanoma cells, it was not until a decade later that the enzyme was connected to lysophosphatidate. The elucidation of the crystal structure by two different groups caused a surge of novel inhibitors.

2. Multiple functions of ATX

In addition to the ability of ATX to stimulate motility, ATX also has ATPase, phosphodiesterase and ATP pyrophosphatase activities [13]. In other words, ATX releases nucleoside-5′-monophosphates from phosphodiester and pyrophosphate bonds [14], which is why it belongs to the family of ENPP (ectonucleotide pyrophosphatase/ phosphodiesterase) enzymes with ENPP1/PC-1, ENPP3/B10 [15], ENPP4 [14,16], ENPP6 [17], ENPP7 [18], and is also named ENPP2 (Table 1). Interestingly, all of the phosphodiesterase catalytic activity of ATX resides in one amino acid, threonine 210. Losing this phosphorylatable residue in the catalytic site results in a loss of phosphodiesterase activity and motility, but not ATP binding [19]. Theoretically, GTP, NAD, FAD, AMP and PPi are all susceptible to hydrolysis by ATX. However, the preferred substrate for ATX is lysophosphatidylcholine (LPC), which it converts to lysophosphatidate (LPA) (Figure 2). Since LPC concentrations in human plasma are greater than 200 μM, this should outcompete the hydrolysis of nucleotide phosphates and pyrophosphate, which are present in much lower concentrations.

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Enzyme	Major Functions	Disease Association
ENPP1 / PC-1	Insulin signaling, glucose import, bone mineralization, immune response	Diabetes, obesity, stroke
ENPP2 / ATX	Vasculature formation, neural development, catalyzes lysophosphatidate production	Cancer, obesity, pulmonary fibrosis, arthritis, asthma, neuropathic pain, liver and colonic disorders
ENPP3 / B10	Basophil activation biomarker	Asthma, allergic reactions
ENPP4	Blood coagulant	Stroke
ENPP5	Putative neural glycoprotein	
ENPP6	Choline-specific hydrolysis of LPC, GPC and SPC	
ENPP7	Alkaline sphingomyelinase	

Table 1. Comparison between enzymes among the Ectonucleotide Pyrophosphatase/ Phosphodiesterase (ENPP) family

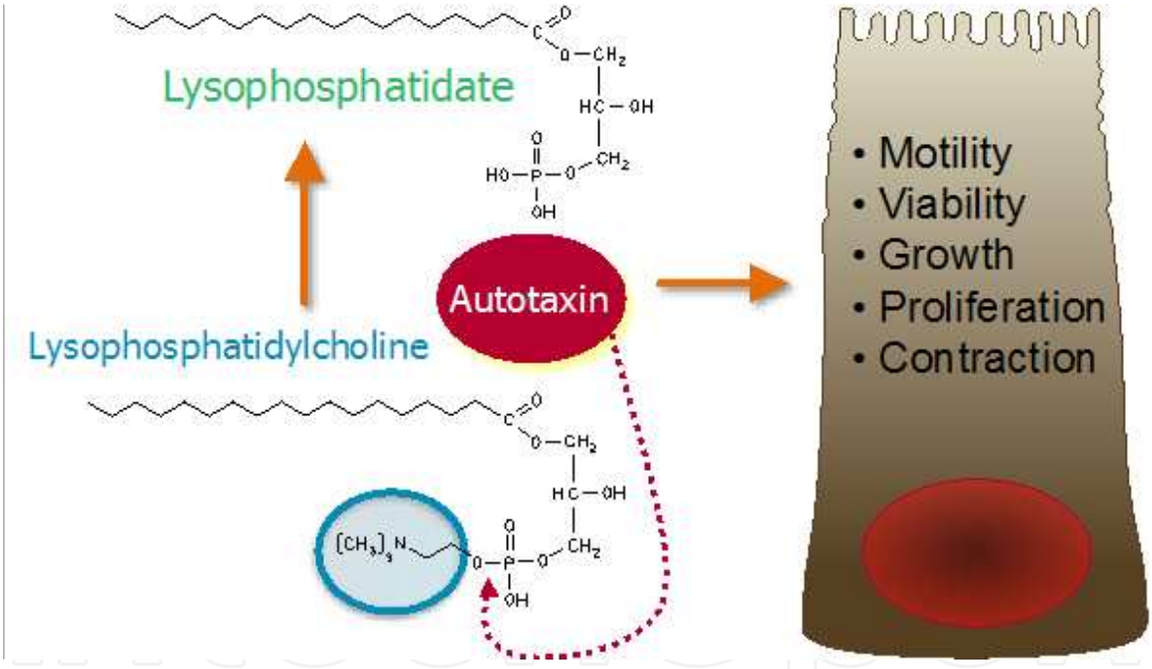

Figure 2. Autotaxin (ATX) catalyzes the biosynthesis of lysophosphatidiate (LPA). Since the lysophosphatidylcholine (LPC) concentrations in human circulation are >200 μM, ATX has abundantly available lipids to produce LPA. ATX hydrolyzes the choline head group from LPC to yield LPA. As a consequence of LPA signaling on cells, a multitude of various responses result, including motility, viability, growth, proliferation and contraction.

The ability of ATX to stimulate motility in melanoma cells can be modulated by chemical methods and genetic engineering. For example, ATX-meditated motility is inhibited by the PI3K inhibitors, wortmannin and LY294002, along with the catalytically inactive mutant of PI3K, PI3KK832R [20]. In addition, *ras*-transformed NIH3T3 cells become more motile and invasive in the presence of ATX. These cells also show enhanced colony formation in soft agar

in vitro and produce significantly larger and metastatic tumors *in vivo* [21]. This suggests that while ATX alone may be insufficient to transform cells to a malignant phenotype, the presence of ATX significantly enhances malignant transformation among 'primed' cells.

3. Role as the main enzyme for the generation of extracellular LPA

The majority of LPA in the circulation is generated by ATX from the abundant LPC (>200 μM in human plasma) in the circulation. In fact, LPC is the major plasma phospholipid and it is bound to albumin [22]. Extracellular LPC is derived from two major routes of metabolism. The first is through the action of lecithin:cholesterol acyltransferase, which is present in plasma high-density lipoproteins. Lecithin:cholesterol acyltransferase, preferentially transfers unsaturated fatty acids from postion-2 of phosphatidylcholine to cholesterol, producing cholesterol ester and a mainly saturated LPC. However, a large proportion of circulating LPC is polyunsaturated [22] and this indicates another route for the production of extracellular LPC. Part of the polyunsaturated LPC is derived from secretion by hepatocytes, but it is possible that other cell types could produce polyunsaturated LPC. Since hepatocytes secrete a large quantity of arachidonoyl-LPC [22-26], it was originally postulated that this might represent a novel transport system for delivering choline and polyunsaturated fatty acids to the brain [22]. Although, this could still be true, we now know that LPC is an important substrate for ATX and that this is the major route for the production of extracellular LPA [27].

This predominant role of ATX in generating LPA is confirmed by circulating LPA concentrations that are 50% of normal levels among ATX heterozygous mice for a null-mutation for ATX [28,29]. Also, ATX inhibition produces a rapid decrease in plasma LPA of >95% [30-32]. The effects of ATX inhibition are more dramatic for the unsaturated species [33]. This is compatible with the substrate preference of ATX for unsaturated and polyunsaturated LPCs [34]. The crystal structures of ATX:LPA complexes show a hydrophobic pocket in the catalytic domain that is slightly U-shaped. This accommodates the kinked acyl chains of unsaturated fatty acids better than the linear conformations of saturated fatty acids [34].

Even though ATX is the major enzyme that generates LPA, other enzymes have a minor role in its biosynthesis. For example, saturated LPA species can also be derived through secretory phospholipase A_2, which hydrolyzes phosphatidate in microvesicles that are shed from cells during inflammation [35] and platelet aggregation [36]. In addition, LPA production by the Group VIA phospholipase A_2 (Ca^{2+}-independent) appears to be involved in the development of prostate cancers [37].

4. Crystal structure of ATX

The elucidation of the crystal structure of mouse and rat ATX revealed that the enzyme is composed of four domains [9,34]. The most important among these domains is the slightly U-shaped catalytic domain, which contains the enzyme's active site, a hydrophobic pocket and

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hydrophobic channel. Although ATX can hydrolyze both nucleotides and lysophospholipid substrates, when the unique hydrophobic pocket engages lysophospholipids, nucleotides are unable to bind to the active site. In addition, lipid acyl chains form further interactions with this pocket that nucleotides do not, which is also why ATX has a higher affinity for lysophospholipids [38]. The unique shape of the catalytic domain also accommodates the kinked acyl chains of unsaturated fatty acids better than the linear conformations of saturated fatty acids [34], which further explains substrate specificity for ATX.

ATX is the only member of the ENPP enzyme family that contains a hydrophobic pocket for lysophospholipids, thus giving it a unique capability over other ENPPs. Interestingly, one amino acid, asparagine 230, is required for ATX to recognize phosphate moieties and produce lysophosphatidate. Mutating asparagine to alanine inhibited all production. [34] Other single-point mutations deep within the hydrophobic pocket are capable of altering binding selectivity and reducing some of ATX's activity [9].

Perhaps the most exciting biological implication arising from the structural resolution of ATX is its proposed role as a directed transporter of LPA. In other words, ATX does not appear to arbitrarily release lysophospholipids from its hydrophobic pocket and channel them into solution. Rather, ATX is hypothesized to directly transfer LPA to its receptors along the plasma membrane. The flat surface of ATX on the side of the channel entrance facing the cell presumably facilitates this purpose [34]. This insinuates a role for ATX as an indirect initiator of cell signaling through its guided transport of an agonist to its receptor. It also further explains early observations of ATX as a motility-stimulating factor, even though it was actually due to LPA.

5. ATX Inhibitors

ATX, as an extracellular enzyme, is a very attractive drug candidate for reducing the abundance of extracellular LPA and subsequent signaling. Indeed, even a transient knockdown of ATX using siRNA is sufficient to significantly reduce melanoma cell viability [2]. Furthermore, the advantage of inhibiting ATX is the attenuation of signaling by all LPA receptors. This is considerably advantageous over the use of individual receptor antagonists because there are at least six and possibly eight confirmed LPA receptors with extensive redundancy. The design of ATX inhibitors has been reviewed previously [39-41] and so we will focus only those inhibitors that show utility *in vivo*.

Among the first ATX inhibitors was L-histidine, which was reported to have an ATX binding affinity (K_i) of about 1 mM *in vitro*, with 10 mM concentrations required to block ATX-stimulated migration in melanoma cells by 90% [42]. L-histidine inhibits ATX non-competitively by chelating cations such as Zn^{2+}, which are required for optimum catalytic rates [42]. Later work showed L-histidine administered intraperitoneally to rats limits thioacetamide-induced liver fibrosis [43]. Although L-histidine is required mM concentrations for activity *in vitro*, the authors cited its importance because it was the first "proof-of-principle" that inhibiting ATX was achievable.

Few studies have focused on inhibiting ATX transcription. However, one study showed that cholera toxin inhibits the proliferation of human hepatocellular carcinoma cells *in vitro* by suppressing the production of ATX through a TNF-α-dependent mechanism [44]. Cholera toxin increases the expression of anti-inflammatory cytokines (IL-4 and IL-10), which suppress ATX mRNA levels [45]. Knockdown of ATX expression with siRNA decreased the effects of cholera-toxin in suppressing the growth of Hep3B and Huh7 HCC cells [44]. Also, oral administration of the anti-inflammatory steroid, prednisolone, decreased serum ATX levels in a dose-dependent manner in patients treated for autoimmune skin diseases [46]. It is uncertain if suppressing ATX activity contributed to symptom relief since tapering the prednisolone dosage caused a rebound in serum ATX levels independently of disease severity. Significantly, the authors proposed that serum ATX levels could be used as a marker of compliance and/or efficacy of steroid therapy [46]. In addition, rifampicin, which is an inhibitor of DNA-dependent RNA polymerase, can be used to lower ATX mRNA transcription in the treatment of cholestatic pruritus. Cessation of rifampicin treatment led to both reoccurrence of pruritus and a rebound of ATX levels to pretreatment levels [47]. All of the treatment options for decreasing ATX expression appear to be effective in only specific diseases. By contrast, ATX inhibitors are designed to block LPA production and therefore this approach should have greater utility in multiple pathologies.

Major criteria for the efficacy of any therapeutic agent is a favorable therapeutic index and good bioavailability and potency. Reports on competitive ATX inhibitors began around 2006. These consisted of carba analogs of cyclic phosphatidate (cPA) with a K_i of approximately 100 nM (Table 2). These compounds inhibited metastasis of melanoma cells, which were injected in the tail vein in mouse models [48] and they inhibited chronic inflammation-induced C-fiber stimulation in rat neuropathic pain models [49]. However, cyclic phosphatidate analogs are also weak agonists of LPA receptors, which limits their utility [39].

Compound	Class	*In vivo therapeutic effects*
cPA	Lipid	• inhibits B16F10 melanoma metastasis in mouse tail vein model [48] • inhibits C-fiber stimulation by chronic inflammation in rat neuropathic pain models [49]
BrP-LPA	Lipid	• reduces MDA-MB-231 orthotopic breast tumor growth in mice [50] • inhibits A549 lung metastasis in engineered 3D mouse xenografts [51] • radiosensitizes GL-261 mouse glioma models [52] reduces collagen-induced arthritis in mice [53]
GWJ-A-23	Lipid	• reduces allergen-induced asthmatic phenotype in ATX-transgenic mice [54] • reduces fibrosis in bleomycin-treated mice [55]
PF-8380	Non-Lipid	• inhibits inflammatory hyperalgesia in rat air-pouch models [30] • radiosensitizes glioblastoma multiforme heteotropic mouse models [56]
ONO-8430506	Non-Lipid	• reduces tumor growth and metastasis in 4T1/Balb/c syngeneic and orthotopic mouse model and is synergistic with doxorubicin [33,57] • reduces urethral tension in rat benign prostatic hyperplasia models [58]

Table 2. ATX inhibitors used to effect a therapeutic response *in vivo*

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A series of lipid-mimetic ATX inhibitors were developed in 2009 based on α-bromophosphonates (BrP-LPA). The *anti*-BrP-LPA isomer had IC_{50} of 22 nM in plasma, which was more potent than for *syn*-BrP-LPA [50] (Table 2). BrP-LPA is also a pan-antagonist of $LPA_{1\text{-}3,}$ which is effective in decreasing to tumor growth in orthotopic xenograft models of breast cancer using MDA-MB-231 cells [50] and in metastasis of A549 lung cancer cells in nude mice [51]. We have also previously demonstrated that BrP-LPA is more effective than dacarbazine in reducing the viability of melanoma cells [59].

In addition to its efficacy against cancer cells, BrP-LPA had a radio-sensitizing effect on the tumor vasculature and delayed tumor growth by 7 days compared to radiation alone in a heterotopic murine glioma model using GL-261 cells [52]. This study was one of the first to demonstrate that ATX inhibitors can potential be used as an adjuvant therapy for cancer. A later report showed that BrP-LPA attenuated disease symptoms by diminishing synovial LPA signaling in a collagen-induced arthritis mouse model. Histological analysis of the joints showed a marked decrease in inflammation and synovial hyperplasia [53].

Several lipid-analogs of LPC, which have been used as ATX inhibitors, have relatively poor bioavailability, mainly because of the hydrophobic acyl tail [41]. This is illustrated by S32826, which is a benzyl phosphonic acid derivative (Figure 3). Despite having a very low nM IC_{50} *in vitro*, the long chain contributes to a very high lipophilicity and it cannot suppress circulating ATX activity for more than a few minutes [32]. Analogs of S32826, such as GWJ-A-23, were developed by shortening the hydrophobic chain and adding α-halo-or α-hydroxy-substituents to increase solubility. However, most of these compounds are less potent inhibitors of ATX compared to S32826 [60], but have been used in pulmonary studies of ATX [54,55]. Hence, much of the recent effort in discovering ATX inhibitors for use *in vivo* has concentrated on compounds that are more soluble and not based on lipid analogs.

There are numerous, small, non-lipid inhibitors of ATX that have been modified to increase potency [40,41]. These inhibitors tend to have better bioavailability because of decreased hydrophobicity and they are unlikely to be rapidly degraded by endogenous hydrolytic pathways [61]. One of these is PF-8380, which is a piperazinylbenzoxazolone derivative that was developed by Pfizer from compound library screening and optimization (Table 2). PF-8380 has an IC_{50} of 2.8 nM against recombinant human ATX and 101 nM for ATX in human whole blood. It was the first ATX inhibitor that was reported to decrease plasma LPA levels *in vivo* for an extended period [30]. In rat air-pouch models, 30 mg/kg PF-8380 inhibited inflammatory hyperalgesia with the same efficacy as 30 mg/kg naproxen, a routinely used nonsteroidal anti-inflammatory drug [30]. This dosage of PF-8380 produced a maximum decrease in LPA concentrations in plasma and also at the site of inflammation. Like BrP-LPA, PF-8380 had radio-sensitizing effects in a heterotopic mouse model of glioblastoma multiforme, delaying tumor growth by at least 20 days [52, 56]. In this study, inhibition of ATX by PF-8380 abrogated radiation-induced activation of Akt and subsequently decreased tumor vascularity and tumor growth [56]. Although we have *repeatedly* tested 30 mg/kg PF-8380 in animal models of melanoma, we have not observed a reduction of tumor growth (results not shown).

We have worked recently with another potent ATX inhibitor, which is a tetrahydrocarboline derivative (ONO-8430506) developed by Ono Pharmaceuticals Ltd. (Patent WO20120052227).

Oral dosing with 10 mg/kg ONO-8430506 suppressed plasma ATX activity as measured by choline release assay in the presence of 3 mM LPC by at least 90% at 6 h after administration in mice [33]. Plasma LPA levels were suppressed, especially the unsaturated species. C16:1-LPA and C20:5-LPA remained non-detectable at 6 and 24 h after ONO-8430506 administration. ONO-8430506 decreased the initial rate of tumor growth and subsequent lung metastasis by up to 60% in a syngeneic orthotopic model of breast cancer in BALB/c mice. This was accompanied by decreased concentrations of unsaturated LPA species in the breast tumors. These findings again confirm that ATX produces most of the extracellular LPA and that decreases in LPA concentrations in tissues mirror the decreases in plasma LPA levels following ATX inhibition [30, 33].

In other work, ONO-8430506 (30 mg/kg/day) decreased intra-urethral pressure and this was ascribed to urethral relaxation [58]. This work demonstrates the potential of ATX inhibition to decrease smooth muscle contraction by LPA. It also shows that ATX inhibition ameliorates urethral obstructive disease, such as benign prostatic hyperplasia.

6. Strategies to identify novel ATX inhibitors

So far, the most common technique for ATX inhibitor discovery and design is to screen libraries of compounds by using assays with ATX substrates such as Fluorescent Substrate-3 (FS-3). Inhibitors are then modified to increase potency. FS-3 is a fluorogenic substrate that is a doubly-labeled analogue of LPC. The fluorophore is quenched through intra-molecular energy transfer. Hydrolysis of FS-3 by ATX increases the fluorescent signal by removing the quencher [62]. The initial studies with this technique identified several inhibitors with an IC_{50} in the μM range [63, 64]. Later work with compounds designed from these studies developed nine pharmacophores for ATX inhibition and the results of these analyses were used to screen the National Cancer Institute's open chemical repository database to prioritize screening efforts [61]. This lead to the identification of several novel compounds with an IC_{50} in the high to low μM range [61, 65].

The identification of the crystal structure of ATX is also enabling structure-activity relationships to be established and more rational-design approaches towards optimizing inhibitor structures are now possible. The first study to report this approach was by Kawaguchi *et al.* who identified a thiazolone derivative from a 81,600-compound library, which inhibited ATX activity with an IC_{50} of 180 nM [66]. The authors proposed a series of side-chain modifications from the crystal structure of ATX complexed with this inhibitor. This led to the synthesis of a derivative compound, 3BoA, which has an IC_{50} of 13 nM [66]. More recent studies by Fells *et al.* combined large library screening results using the crystal structure of ATX with virtual screening tools to discover additional novel ATX inhibitors [67, 68]. This technique identified a common aromatic sulfonamide structural motif among potent inhibitors that targets the hydrophobic pocket of ATX. This accommodates the hydrocarbon tail of LPC/LPA [67, 68]. Similar techniques are expected to lead to the rapid development of ATX inhibitors for subsequent testing and development *in vivo*.

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7. Physiological functions of ATX and LPA signaling

7.1. Vasculature system

Although studies *in vitro* detected no difference in the growth rate of *ras*-transformed NIH3T3 cells with or without ATX in culture, the growth rates in animals were significantly different, which suggested a role for ATX in blood vessel formation [21]. Indeed, Matrigel plugs of cells with ATX displayed extensive micro-aneurysms filled with red blood cells and more tumor cells, further corroborating this role. Furthermore, the ability of ATX to stimulate blood vessel formation in Matrigel plugs after 6 days was equivalent to VEGF, thus solidifying ATX as an angiogenesis-stimulating factor in the circulation [69].

Developmentally, the expression of ATX is indispensible. Beginning at 8.5 days, ATX expression is detectable in the early mouse embryo, within the floor plate of the neural tube [70]. Knocking out of ATX in mice causes embryonic lethality around 9.5 – 10.5 days with embryos exhibiting open, kinky neural tubes [28,71]. Although heartbeats are detected until 10.5 days *in utero*, contractions are weak, irregular and clearly abnormal. In addition, even the yolk sacs of ATX knockout embryos are irregular with a complete absence of blood vessels. [72] The role of ATX in vascular and neural development is also observed in zebrafish [72, 73]. ATX regulates the differentiation of oligodendrocytes in the hindbrain [74] and the correct left-right asymmetry for normal organ morphogenesis through Wnt-dependent pathways [75]. Taken together, these results definitively prove that the absence of ATX results in severe defects of the vasculature system and embryonic lethality. Organisms cannot develop without functional ATX expression.

Other groups have explored the parallel correlation by investigating the vasculature system when ATX is overexpressed in transgenic mice. Interestingly, ATX transgenic mice have an unusual susceptibility to bleeding and impaired platelet-dependent thrombus formation after injury [10]. Further studies have confirmed an important role for ATX in platelet activation through the production of LPA [76]. In addition, ATX binds to platelet β1-and β3-integrins, which localizes ATX to the cell surface of the appropriate microenvironment [11].

8. Adipose tissue regulation

An alternative, yet highly interesting function of ATX *in vivo* occurs in adipogenesis, obesity and the regulation of glucose homeostasis [77]. Foremost, ATX is expressed and secreted by adipose tissue. Its expression is also significantly upregulated during pre-adipocyte differentiation into adipocytes (adipogenesis) as well as in the adipose tissue of genetically obese diabetic mice [78]. In support of this finding, transgenic ATX mice accumulate more fat than littermates when they are fed a high-fat diet and thus, are more susceptible to diet-induced obesity [79]. Other work showed that ATX mRNA is upregulated in differentiating adipocytes but downregulated in hypertrophied adipocytes from obese mice [80]. Further, in humans, serum ATX levels and ATX mRNA expression in subcutaneous fat were negatively correlated

with body mass index [80]. However this does not appear to be true if the subject is diabetic. Instead, these patients tend to have higher serum ATX levels [80]. In regards to the regulation of glucose metabolism, LPA produced by ATX is able to dose-dependently inhibit glucose-induced insulin secretion [77, 81]. Furthermore, knocking out the expression of ATX in adipose tissue results in a mouse with improved glucose tolerance [82].

9. Wound healing, tissue remodeling and inflammation

ATX and LPA facilitate critical processes necessary for skin re-epithelialization and wound healing. For example, among blister fluids, both ATX and LPA are produced and detected, originating *de novo* in the blister fluid and not from plasma. LPA is a potent activator of platelet aggregation and promotes keratinocyte migration, proliferation and differentiation. Thus, ATX and LPA facilitate critical processes necessary for skin re-epithelialization and wound healing [83]. ATX expression and LPA production are increased in rabbit aqueous humor following wounding by corneal freezing [84].

The range of physiological functions requiring ATX is quite diverse. For example, ATX and LPA signaling are involved in luteal tissue remodeling of regressing corpora lutea in rat ovaries. This occurs by recruiting phagocytes and proliferating fibroblasts, which are ultimately the factors involved in remodeling [85]. Other roles for ATX include hair follicle morphogenesis [86], bone mineralization [87] and myeloid differentiation in human bone marrow [88].

Another unique role of ATX occurs in response to oxidative stress in microglia, whereby ATX expression is increased. This protects microglia cells from damage by H_2O_2 and this effect is partially reversed by the mixed $LPA_{1/3}$ antagonist, Ki16425 [89]. Microglia cells overexpressing ATX show suppressed production of the pro-inflammatory cytokines, TNF-α and IL-6, and increases in the anti-inflammatory cytokine IL-10 upon treatment with lipopolysaccharide [90]. ATX is expressed in high endothelial venules in lymph nodes and other secondary lymphoid tissues [91]. This mediates lymphocyte extravasation, a process required for maintaining immune homeostasis [92, 93]. However, in chronically inflamed tissues, ATX mediates lymphocyte trafficking and increases cytokine production in response to repeated micro-injuries and incomplete tissue repair [94-96].

Interestingly, the catalytic activity of ATX has a dualistic role in wound healing. In this way, an ATX-like enzyme, SMaseD, is responsible for the pathology associated with venomous poisons through its dermonecrotic and hemolytic activities [97, 98]. In other words, the aberrant over-production of LPA by ATX cultivates an inappropriate immune response, similar to a wound that never heals, whereby overabundant inflammatory cytokines and chemokines are released. The damage is manifested in several ways, including the presence of severe dermonecrosis with blackened or missing skin appearing at the wounded site. The dermonecrosis can occur after envenomation by either *Loxosceles reclusa,* the brown recluse spider [97], or *Hemiscorpius lepturus,* a venomous scorpion [98]. Intriguingly, susceptibility to SMaseD is conferred by the LPA_1 receptor [99], suggesting a possible role for LPA receptor antagonists in this pathology.

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As mentioned above, one of the main functions of ATX in adults is to repair damaged tissue. ATX is secreted in this situation partly in response to inflammation and the release of inflammatory cytokines. In normal wound healing, the production of LPA by ATX causes cells to migrate into the area of damage to effect wound repair and the formation of new blood vessels. In cases where the inflammation is not resolved, the process can result in tissue damage and fibrosis as in rheumatoid arthritis, atherosclerosis, organ fibrosis, diabetes and even obesity [100]. Cancer can be added to this list since it has been likened to "a wound that does not heal" [101]. The role of inflammatory cytokines in tumor progression [102-106] explains why inflammatory bowel disease and viral hepatitis can progress to cancer [107].

10. ATX in malignancy: Metastasis and angiogenesis

ATX is among the top 40 upregulated genes in metastatic cancer [108] and this is explained by the effects of LPA, which signals through at least six and putatively eight G-protein-coupled receptors. Through these receptors, LPA stimulates cell motility, cell survival/viability, cell proliferation, morphological changes, contraction, wound healing and invasion [109-118]. LPA achieves these effects by signaling through the relative activations of phosphatidylinositol 3-kinase (PI3K), $ERK_{1/2}$, mTOR, Ca^{2+}-transients, Rac, Rho and Ras [119].

The involvement of ATX and LPA in tumor progression affects multiple malignant processes and stages of tumor progression. For example, LPA increases the production of vascular endothelial growth factor, which stimulates angiogenesis [51, 120], a process required for tumor growth beyond 1 mm. However, in order for tumors to arise at all, tumor suppressors must be made ineffective. LPA levels can rise to 10 μM in the ascites fluid from advanced ovarian cancer patients [121]. Interestingly, LPA also decreases the abundance of the tumor suppressor, p53 [122], thus increasing cancer cell survival and proliferation, even in the presence of actinomycin D.

Pre-clinical models of disease and clinical pathology provide insight into the role of ATX and LPA receptors in cancer. For example, transgenic multiparous mice designed to overexpress ATX, LPA_1, LPA_2 or LPA_3 in mammary epithelium develop spontaneous metastatic mammary tumors as they age [123]. Women who express high levels of LPA_3 receptors in epithelial cells, or ATX in stromal cells, have larger breast tumors, nodal involvement, and higher stages of disease [124]. Since many early stage breast cancer patients are able to be cured using current treatment modalities, this suggests that the presence of ATX and/or LPA receptors has the ability to alter outcomes of malignancy.

The involvement of ATX and LPA in tumor progression can be understood in terms of a dysfunctional wound healing response. As mentioned above, one of the main functions of ATX in adults is to repair damaged tissue. ATX is secreted in this situation partly in response to inflammation and the release of inflammatory cytokines. In normal wound healing, the production of LPA by ATX causes cells to migrate into the area of damage to effect wound repair and the formation of new blood vessels. Cancer can be considered to be a case of unresolved inflammation and it has been likened to "a wound that does not heal" [101]. In

fact, inflammation is now considered to be one of the "Hallmarks of Cancer" [125]. The secretion of ATX and increased LPA signaling should now be included as one of the inflammatory factors that drives tumor progression.

The role of ATX and LPA in this process is well illustrated in the case of work with mouse models of breast cancer. Most breast cancer cells do not themselves express ATX, but rather this is produced by fibroblasts within the breast tissue or by the surrounding adipose tissue [33] (Figure 3). The development of the breast tumor causes the release of inflammatory cytokines, which stimulate fibroblasts and adipose tissue to secrete ATX in a syngeneic orthotopic mouse model of breast cancer [33, 107]. This is part of a vicious cycle since LPA in turn stimulates the production of inflammatory cytokines [126-128]. This inflammatory cycle can be effectively blocked in a mouse by inhibiting ATX activity with ONO-8430506, which results in about a 60% decrease in tumor growth and lung metastasis.

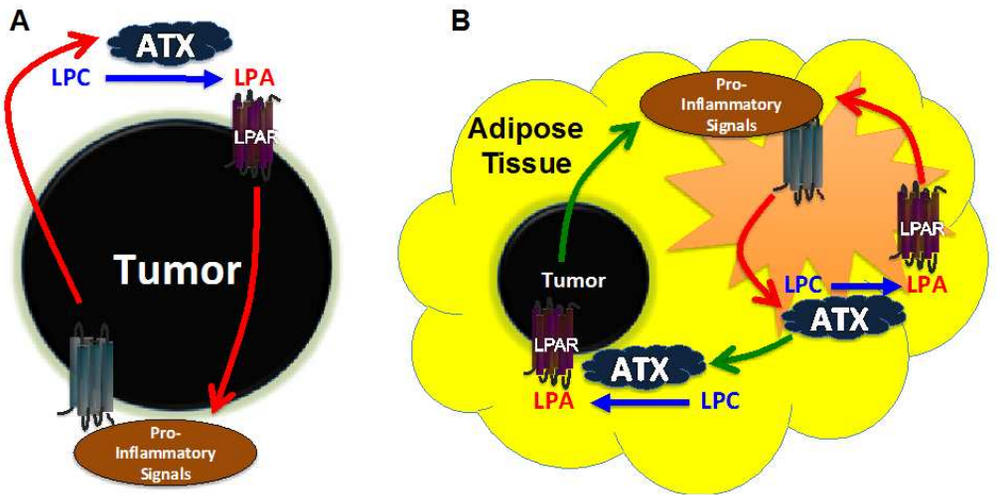

Figure 3. Proposed inflammatory-mediated models of ATX secretion. A) In cancer cells that overexpress ATX such as thyroid cancers, neuroblastomas and melanomas, autocrine-secreted ATX produces LPA, which signals through LPA receptors (LPARs) on the cancer cell surface. This signaling further increases the production of pro-inflammatory signals including growth factors, cytokines and chemokines. These molecules in turn can signal through specific receptors to increase ATX production. B) In breast cancer, a paracrine model of ATX secretion is possible since breast adipose tissue secretes high levels of ATX, whereas breast cancer cells normally produce negligible ATX. As the tumor grows, pro-inflammatory signals secreted by the tumor create an inflammatory environment within the surrounding adipose tissue (green arrows). This signaling increases ATX secretion and LPA production, which in turn can establish an autocrine feedback loop of increased pro-inflammatory signaling and ATX production (red arrows) within the adipose tissue. Increased ATX and LPA production can in turn contribute to tumor progression (green arrows). A combination of both autocrine and paracrine production of ATX is also possible when cancer cells produce significant quantities of ATX.

Although breast cancer cells do not do not express significant levels of ATX activity, this is not typical of other tumors where ATX activity is expressed in the cancer cells themselves. These cancers include thyroid [129], neuroblastomas [71, 130, 131] and melanomas [132-134]. However, in the case of melanomas, their normal predecessor cells, melanocytes, do not express ATX. Thus, the data suggests that ATX expression is acquired during the transition to malignancy in melanoma [2]. The secretion of inflammatory cytokines by cells in the tumor also produces a vicious cycle of ATX secretion and LPA production, which is a driving force

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in tumor progression [107]. In the case of breast tumors, we propose that the ATX come from surrounding adipose tissue, whereas in thyroid tumors, neuroblastomas and melanomas, ATX is secreted by the cancer cells (Figure 3).

11. ATX in chemo-resistance

Another important role of ATX expression and LPA signaling in malignancy occurs during the acquisition and manifestation of chemo-resistance. LPA facilitates chemo-resistance to the cytotoxic effects mediated by Taxol [119, 135, 136], doxorubicin [57], actinomycin D [122] and carboplatin [137]. These effects are mediated by LPA partly through activation of survival and viability pathways, such as ERK and PI3K. In addition, we previously demonstrated that LPA signaling does not encompass the entire molecular mechanism and there are other proteins, like the Regulators of G-protein Signaling proteins, which play a more dominant role [138]. Indeed, in the absence of appropriate Regulators of G-protein Signaling proteins, cells exposed to LPA have increased capacity to acquire chemo-resistance.

As chemo-resistance is a complex process, there are other molecular mechanisms involved. For example, among chemo-resistant cells, increased expression of multidrug resistance transporters enables toxins, like chemotherapeutic drugs, to be exported out of cancer cells. This is particularly problematic in the case of renal cell carcinomas, for which cytotoxic chemotherapy is largely ineffective, but also occurs widely in malignancy.

Recent work shows that the activation of PI3K by through LPA_1 receptors increases the stability of the transcription factor Nrf2, which increases the expression of antioxidant genes and multidrug resistant transporters [57]. The expression of antioxidant genes protects cancer cells against the oxidative damage caused by chemotherapeutic agents. Also, the expression of multidrug resistant transporters enables toxic oxidative products and chemotherapeutic drugs to be exported out of cancer cells. These effects explain why inhibiting ATX activity and blocking LPA signaling improves the efficacy of doxorubicin as a chemotherapeutic agent [57]. Thus blocking ATX activity can provide a novel adjuvant therapy for improving the efficacy of existing chemotherapeutic agents.

ATX inhibition could also have a beneficial effect as an adjuvant for improving the effects of radiotherapy as discussed above. This is possible since LPA, through activation of LPA_2 receptors, also protects against radiation-induced cell death. This action depends on the depletion Siva-1, which is a pro-apoptotic signaling protein [139].

The function of ATX in aggravating resistance to chemotherapy and radiotherapy can be understood in terms of the vicious cycle of inflammation caused by repeated bouts of therapy as described above [140] (Figure 3). Cancer therapy itself causes damage to the tumor and surrounding tissue, which responds by producing inflammatory cytokines resulting in increased ATX production [107]. This explains why blocking this cycle by inhibiting LPA formation can improve the sensitivity to chemotherapy by attenuating the effects of increased Nrf2 expression.

12. ATX in melanoma

Although accumulating studies suggest that inhibiting ATX activity could provide a novel adjuvant therapy for improving the efficacy of existing chemotherapeutic agents, we have previously demonstrated a role for ATX inhibitors as monotherapy against advanced cutaneous melanoma [2,48,59]. After injecting B16F10 metastatic melanoma cells into the tail veins of C57/Bl6 mice, we observed a significant reduction in the number of lung nodules, which represent metastatic melanoma tumors, after treatment with a phosphonothionate analogue of carba cyclic phosphatidic acid, thio-ccPA 18:1 [2]. This compound was synthesized for improved metabolic stability and activity, based on our previous results [48]. In addition to being an inhibitor of ATX, thio-ccPA 18:1 is a direct antagonist of LPA_1 and LPA_3 receptors [141].

As mentioned previously, melanomas are notoriously resistant to chemotherapy. In light of the role of ATX/LPA signaling in melanoma, perhaps it should not be surprising that melanoma cells, which produce high quantities of ATX, are resistant to chemotherapy, since excessive LPA signaling contributes to this phenotype. Only a few chemotherapy agents are approved options against melanoma, these include dacarbazine and temozolomide. Thus, we compared these single agents against both the anti-BrP-LPA and the mixed diastereomers BrP-LPA on the viability of MeWo melanoma cells. Indeed, both BrP-LPA compounds were more effective single-agents at 10 μM and 100 μM than either dacarbazine or temozolomide, at concentration ranging from 10-1000 μM [59]. This suggests that targeted approaches against ATX in melanoma have potential and further results will be reported in due time.

Besides cutaneous melanoma, in a study on uveal melanoma, ATX was the only gene among 32 candidate genes whose expression was sufficient to distinguish classes representing metastasis and prognosis. Paradoxically, "underexpression" of ATX correlated with poor prognosis and metastatic death among 27 samples [132]. Based on the discussion provided above it is tempting to propose that melanocytes evolved to survive solar ultraviolet radiation and simultaneously provide protection to neighboring cells by producing ATX and thus providing LPA. However, with repeated DNA damage and incomplete repair from excessive UV radiation, melanocytes are malignantly transformed into melanoma cells. At this point, the increased production of ATX and signaling by inflammatory cytokines, which are meant to facilitate repair, could be subverted into promoting cancer progression.

13. Summary and conclusions

Advanced metastatic melanoma is an incurable disease in dire need of additional therapeutic options. Although many newly targeted inhibitors have extended the life of patients with *BRAF* mutations, they do not achieve cure due to chemo-resistance, and they are not applicable to patients with Wild-type B-Raf. Thus, additional therapeutics are desperately needed to treat this growing population. Herein we have summarized the current state of ATX inhibitors and what we currently know about the role of ATX in melanoma and malignancy.

The original ATX inhibitors had little utility *in vivo* because of the very low bioavailability. However, as described above, potent ATX inhibitors are now being developed, which are

effective *in vivo* for longer than 24 h. These inhibitors appear to be well tolerated by animals and the next stage is to take such inhibitors through Phase 1 clinical trials. These ATX inhibitors should be effective in improving the outcomes for which the ATX/LPA axis is involved. These include various forms of cancer whereby an ATX inhibitor could be used as a monotherapy or as an adjuvant to improve existing chemotherapies or radiation treatment. The ATX inhibitors should also be effective in improving the treatment of a variety of inflammatory conditions. These compounds deserve further examination.

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