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REVIEW

Autotaxin

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Abstract Autotaxin is a protein of approximately 900 amino acids discovered in the early 1990s. Over the past 15 years, a strong association between cancer cells and autotaxin production has been observed. Recent publications indicate that autotaxin and the capacity of cancer to metastasise are intimately linked. The discovery of new molecular targets in pharmacology is a mixture of pure luck, hard work and industrial strategy. Despite a crucial and desperate need for new therapeutic tools, many targets are approached in oncology, but only a few are validated and end up at the patient bed. Outside the busy domain of kinases, few targets have been discovered that can be useful in treating cancer, particularly metastatic processes. The fortuitous relationship between autotaxin and lysophosphatidic acid renders the results of observations made in the diabetes/obesity context considerably important. The literature provides observations that may aid in redesigning experiments to validate autotaxin as a potential oncology target.

Keywords Autotaxin · NPP-2 · *Lyso*phosphatidic acid · *Lyso*phosphatidylcholine · Cancer · Metastasis · Obesity · Enzymatic assays

Abbreviations
lpa *Lyso*phosphatidic acid
lpc *Lyso*phosphatidylcholine

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Introduction

Autotaxin (ATX) is a recently discovered phospholipase D that transforms lysophosphatidylcholine (lpc) into lysophosphatidic acid (lpa). The protein was described as a motility factor for about 10 years until its catalytic capacity was understood. By the time the present work took form, several very relevant reviews had been published, particularly regarding the use of ATX inhibitors in inhibiting metastases [1, 2]. Nevertheless, we present a different angle on ATX, one that is more biochemistry- and industry-oriented.

Autotaxin as a motility factor (1992–2001)

Cancer cell motility factors had been known for some years [3] when Liotta's group identified and cloned one from human melanoma cell line A2058 in 1992 [4]. The A2058 cell line is a particularly rich source of autocrine motility factors [5]. Liotta's group reported the purification and characterisation of the 125-kDa protein they named autotaxin. This factor was also shown to stimulate cell migration at concentrations below 1 nM. Interestingly, Liotta hypothesised the existence of an ATX receptor, possibly G protein-coupled, because this cell migration was sensitive to pertussis toxin. It was later discovered that the receptors indeed existed, but were lpa receptors; lpa is the product of an enzymatic reaction catalysed by ATX (see the following section). The protein was cloned and exhibited common features of the phosphodiesterase family [6] (see also the review by Goding et al. [7]). The protein was then renamed ENPP2 by analogy with the various members of the phosphodiesterase family of enzymes [8]. ATX has been purified additionally from

many cancer cell lines, including those originating from tetracarcinoma and breast cancer. It was demonstrated that ATX could catalyse phosphodiesterase, pyrophosphatase and ATPase activities, although with a low catalytic capacity [9]. Several reports described PD1α and ATX as alternative splicing products of the same gene [10], a feature that was later confirmed by our laboratory when we cloned, expressed and characterised the three main isoforms of ATX [11]. As a motility factor, ATX was screened for its role in cell migration [12, 13], including that of cancer cells, already suggesting a major role for this protein in the regulation of cell behaviour. The motility capacity of ATX was tentatively linked to its phosphate hydrolysing activity with key endobiotic metabolites in cells, such as NAD, AMP or GTP, including a possible auto-phosphorylation process on Thr^{210}, the key amino-acid of the ATX catalytic site [14]. Photo-affinity attachment sites were described at the critical area of Thr^{210} and a slightly more distant region starting at Tyr^{318} in rat ATX [15]. It was speculated that the nature of this phosphorylation and auto-dephosphorylation site(s) was a possible trait of the protein phosphatase capacity of ATX, in line with its role in motility.

Autotaxin as a phospholipase D (2002—present)

Enzyme purification

Starting from three different biological materials, three groups purified a lysophospholipase D around the same time. The first groups who published identified this activity in human plasma [16] and fetal bovine serum [17]. We started with conditioned media from mouse adipocytes and found the same activity catalysed by the same enzyme [18]. These discoveries shed new light on all previous ATX studies. Autotaxin then received the E.C. number 3.1.4.39 (www.expasy.org). Because the main catalytic activity of ATX is the production of lysophosphatidic acid, it became clear that most, if not all, previously described roles were mediated by this phospholipid through its interaction with a family of seven transmembrane domain, G-coupled receptors (see for reviews [19–21].

The ATX gene and isoforms

The ATX gene is located on chromosome 8 at position 8q24.1 in humans and chromosome 15 in the mouse. The structure of the gene is of high complexity; it contains 27 exons and could potentially have a high number of alternative splicings. The ATX isoforms in human and murine tissues were described previously [6], but they were not compared. Giganti et al. [11] performed such a comparison by cloning, expressing, purifying and biochemically characterising the isoforms. Interestingly, no major differences were found at any stage between the two major isoforms (β and γ) (Fig. 1a).

The ATX isoforms are differentially expressed. High levels of ATX β mRNA expression are detected in peripheral tissues, whereas lower expression levels are observed in the central nervous system. In contrast, the highest levels of mRNA expression for the ATX γ variant are detected in total brain, whereas significantly lower expression levels are observed in peripheral tissues. In the central nervous system, ATX γ is expressed at similar levels across all brain regions studied. Among the three isoforms, ATX α exhibits the lowest expression levels in both the central nervous system and peripheral tissues. All these expression levels have been confirmed by qPCR experiments [11]. The α isoform is very unstable because it contains an extra exon (exon 12) compared to isoforms β and γ. The expression of this exon creates a cleavable sequence in which a protease site appears (KVAPKRR) corresponding to position 341 of the human isoform. Interestingly, we found that the β and γ isoforms are easily expressed in at least four transgenic systems (bacteria, Chinese hamster ovary cells, COS and Sf9 insect cells). The α isoform seems to be poorly expressed in any of these systems under identical conditions. The cleavage has been observed in all host systems tested (mammalian, bacteria and insect), suggesting that it is due to a non-specific protease. The shortened protein is devoid of enzymatic activity because the core of the catalytic site (Thr^{210}) is in the cleaved fragment. Under non-reducing conditions, the apparent molecular mass of the α isoform remains in the

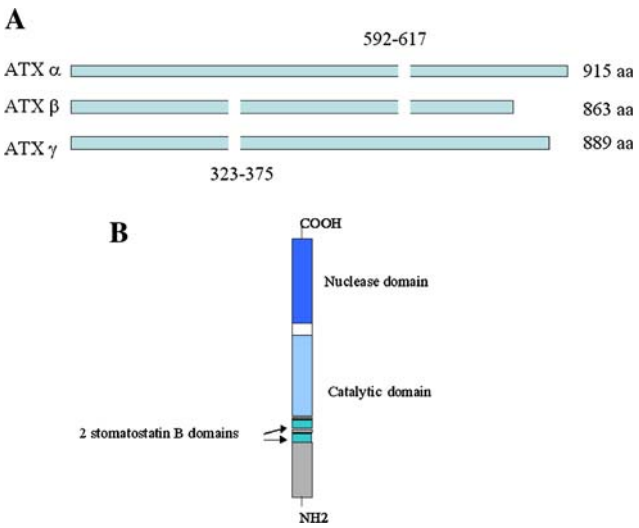

Fig. 1 Schematic representation of three main autotaxin (ATX) isoforms: α, β and γ (**a**) and the ATX protein(**b**) [6]

100 kDa range, whereas, under reducing conditions, it decreases to 66 kDa, which is in line with the predicted cleavage site location and suggests that one or several SDS-resistant disulfide bond(s) remain intact upon protease treatment. Interestingly, this particular isoform might correspond to the one described by Pamuklar et al. [22]. Pamuklar constructed a model driving the expression of ATX under the control of the human antitrypsin promoter and noticed that mice over-expressing ATX in blood had an immuno-reactive protein of roughly 70 kDa on Western blot. Although we reported the three main isoforms [11], we isolated at least two other, shorter isoforms with no predominant characteristics (Cogé, Ferry and Boutin, unpublished).

The ATX protein

At the time of our work on ATX, the key features of the protein were already known due to its description in the historical purification of PD1α and as a motility factor. The catalytic activity of ATX had been reported for phosphodiesterase, pyrophosphatase and ATPase activities, but not for phospholipase D. The ATX protein has an intramembrane domain, two cysteine-rich somatomedin B-like domains located adjacent to a hydrophobic domain containing an RGD tripeptide motif that may be involved in cell–extracellular matrix interactions, a phosphodiesterase domain, the catalytic domain of lysophospholipase D and a nuclease-like domain located at its C-terminus [6] (Fig. 1b). It was suspected that the enzyme is synthesised as a pre-pro-enzyme from which a 27-amino-acid peptide signal is cleaved. An octapeptide from the N-terminus is then removed by pro-protein convertases, and the mature ATX is secreted [23]. The issue with this description is that the intramembrane domain was probably never in a transmembrane configuration. Indeed, it has been reported that ATX is not expressed on the surfaces of ATX-transfected cells or cells secreting ATX [24]. Later studies from Pradere et al. [25] also showed that *N*-glycosylation of Asn^{53} and Asn^{410} is essential for the secretion and activity of ATX, at least in adipocytes. An earlier publication showed that ATX is an *N*-linked glycoprotein, but the sugar moieties are not needed for its stimulation of cellular motility [26]. Because this motility depends on lpa production, this paper indirectly showed that *N*-glycans are not essential for catalytic activity, in contrast to what was reported elsewhere [25].

More detailed structural information will soon become available, as the purification of the extracellular domain of ATX has been described in detail [27] with this purpose in mind. As of today, only a model of the 3D structure of ATX has been suggested by analogy with NNP1 and NNP3 [28–30].

The main catalytic characteristics of ATX include its marked specificity for LPC species of high molecular weight (16:0–18:2) and for nucleosides [11, 16]. Furthermore, ATX is also capable of hydrolysing sphingosylphosphorylcholine, opening up new potential roles of ATX in other cellular functions [31]. Gijsbers et al. [32] also demonstrated that the hydrolytic activities of ATX, both phospholipase D and phosphodiesterase, are catalysed by a single site, a feature so stringent that Thr^{210} replaced by an Ala not only renders the enzyme inactive, but, once knocked-in in mice, it led to the lethality of homozygous animals [33]. Another publication from the same group studied the specificity features of ATX at the sequence level, in regards to nucleotides and lysophospholipids. In particular, the group pinpointed the importance of a G/FXGXXG motif in the area of the catalytic site for ATX activity [34], which is important for metal binding but not substrate specificity. Finally, a recent report also addressed the relationship between the amino acids proximal to the catalytic site and their roles in catalytic activity and specificity using point mutations: histidine to glutamine at positions 226, 434 and 420 [35].

Further work described the three isoforms of ATX [11] and their respective characteristics. The manipulation of nearly pure proteins is often a source of complications. Indeed, pure proteins sometimes have the capacity to precipitate, aggregate or bind to the dish, tube or plate plastics. In our studies, ATX behaved in such a way. Once pure, this protein did not remain in solution, but tended to form huge aggregates. A close and complete study was performed to approach and solve this problem [11]. By adding various constituents to the ATX solution, conditions were found under which the protein stability and solubility were enhanced [18]. These additives had a significant effect on the kinetic and catalytic constants of the enzyme. Therefore, taking advantage of the many available additives useful in crystallography, a mixture containing, in particular, 2-methyl-2,4-pentanediol was tested to help maintain the protein in solution and keep it stable without altering its affinity for the substrate [36].

Biochemical characteristics of ATX

The characteristics of the ATX isoforms are not known because most of the data previously available in the literature were gathered from a form purified from human plasma [17]; undetermined isoforms were cloned from rat [37], mouse [38], humans [39] or hen egg white [40], or homogenates of various origins, mainly rat liver [41] and rat brain [42]. Interestingly, the γ isoform was recently cloned by Pamuklar et al. [22].

To describe the enzymatic characteristics, the pH and temperature dependence of ATX were evaluated. A peak of activity at pH 8 and an optimum temperature activity of

approximately 40°C were obtained. Notably, even at 60°C, ATX was still able to catalyse a significant portion of its maximal activities [11].

Autotaxin, as measured by nucleotide pyrophosphatase activity, is activated by divalent cations [43]. It has been known for a long time that cations can stimulate enzymatic activities through various mechanisms. Early publications indicated that lyso-PLD from rat liver requires Mg^{2+} and is inhibited by Zn^{2+}, Mn^{2+} and high concentrations of Ca^{2+} (25 mM) [44]. In contrast, Mg^{2+} (5 mM) has little stimulatory effect in rabbit kidney [45]. Instead, the rabbit kidney lyso-PLD requires Ca^{2+} (5 mM) for normal function. Based on these results, the microsomal lyso-PLDs were divided into Mg^{2+} and Ca^{2+}-dependent enzymes. The lyso-PLD in rat plasma requires a metal ion for optimal activity [46], and Co^{2+} was the most effective, followed in decreasing order by Zn^{2+}, Mn^{2+} and Ni^{2+}. The lyso-PLDs from human plasma and adipocytes are likewise activated by Co^{2+} [47]. The mechanism of cation effects on lyso-PLD activity is not well understood. Co^{2+} enhances the phosphodiesterase activity of recombinant human and rat ATX as well as purified plasma lyso-PLD [16]. Furthermore, the PDE activity of ATX is enhanced by Ca^{2+} and Mg^{2+} in a concentration-dependent manner. For ATX, cations might act either to stabilise the structure or as part of the catalytic centre [48]. In addition, cations protect ATX from thermal denaturation and proteolysis [43]. Cations may exert their stimulatory effect by interacting with a region other than the EF-hand loop region in the ATX structure. Note that, although Co^{2+} stimulates ATX activity, it is not absolutely required.

Measuring enzyme activity

Some of the various assays used for ATX measurement are shown in Fig. 2. The question of natural substrates of ATX has been addressed by Gijsbers et al. [32], particularly the differences found among NPP1, 2 and 3. Among those three isoforms, only NPP2 (ATX) is able to catalyse phospholipase D activity, and the capacity of ATX to catalyse phosphatase-like activity seems to be low, particularly regarding ATP and other nucleotides, such as thymidine 5′-monophosphate [32]. Furthermore, purified ATX may also cleave sphingosylphosphorylcholine to yield sphingosine-1-phosphate, a lysolipid with potent bioactivities [31]. Many of the studies on ATX have been run with the phosphodiesterase artificial substrate, *para*-nitrophenylphosphonate, an easy-to-use compound, but ATX is a mediocre phosphodiesterase, having much better affinity for lpc than for nucleosides [11, 49]. However, this might favour the finding of poorer inhibitors as the screening versus a poor substrate results in such a selection of poorly active compounds. The most direct method, using thin layer chromatography (TLC) after liquid/liquid extraction and using labelled lpc, certainly remained the most appropriate, though the most time-consuming, method. Although considerable progress has been made in the area of quantification and run-out in TLC assays, we

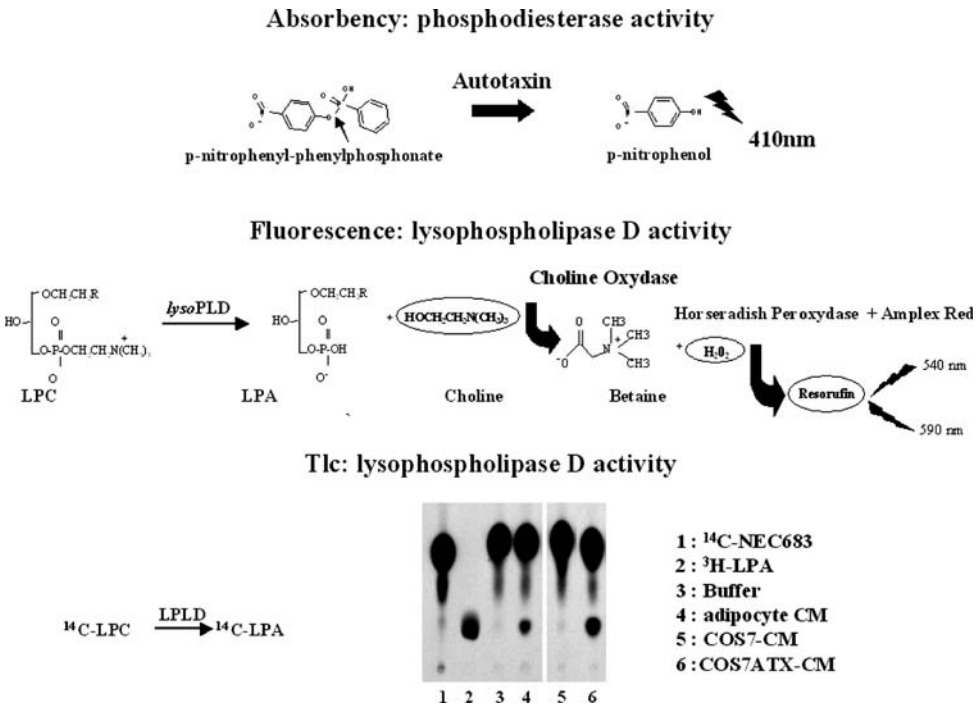

Fig. 2 The three main methods of measuring autotaxin (ATX) activities: absorbency for phosphodiesterase activity and fluorimetric and thin layer chromatography for lyso-phospholipase D. The use of FS-3, a fluorogenic phospholipid, as an ATX substrate is not pictured [50]

found it difficult to turn this assay into a high-throughput screening process. A third elegant method was assessed by Umezu-Goto et al. [17] that took advantage of the possibility of coupling the release of choline from the ATX reaction with a fluorescent signal. Choline is oxidised by choline oxidase, which is added to the incubation, leading to the production of H_2O_2, which in turn reacts with horseradish peroxidase to form resorufin from Amplex Red. The fluorescence of resorufin gives a strong signal that can easily be measured. We found it difficult to use this system for measuring ATX activities in complex media, but it was a perfect one to use with purified proteins. Our assessment of this method using plasma or serum led to a strong aspecific signal, independent of the presence of ATX.

A fourth system has been reported: the use of a synthetic fluorescent phospholipid substrate (FS3) [50]. The substrate molecule was derived with both a fluorescent probe (bodipy in place of the lipid moiety) and a quenching compound (dabcyl) at the far end of choline. The molecule is not fluorescent until cleavage occurs. This compound permits the assay to be clearly simplified, as no extraction and TLC are needed. Unfortunately, our own use of that compound led to the observation that the substrate is cleaved off in complex media, leading to extra peaks in HPLC that are not due to ATX cleavage (Giganti, Ferry, Boutin, unpublished results). When expressed in Sf9 insect cells, we found that control cell conditioned media (i.e. from cells transfected with empty vector) catalyses activity when measured with this system, whereas no activity was measured with the TLC assay or phosphodiesterase assay (i.e. probably showing the absence of endogenous insect ATX). A similar attempt was reported by Van Meeteren et al. [39] using a FRET compound (CPF4) [51]. Therefore, and unfortunately, the question of a completely reliable, easy-to-use ATX assay is still unanswered.

Another important concern is about the measurement of ATX activity in blood. Hen ATX was reported to be inhibited by ovalbumin, even when this protein was "fatty acid-free" [40]. It has been believed for some time that mammalian ATX is inhibited by albumin. We demonstrated that this was not the case unless albumin was not lipid-free [11]. Indeed, ATX inhibition by fatty acids was previously documented, as well as its inhibition by high concentrations of the product of the reaction (i.e. lpa). Thus, it was surprising that serum can be used as a biological source to measure ATX activity, a fact that limits the measurement. For instance, the initial ATX activity in the first process of ATX purification from serum recorded activities as low as 12 pmol/(min mg protein) [16] when starting from 65 g of protein. The purification, encompassing six chromatographic steps, ended with the purification and identification of ATX after an approximate 50,000-fold enrichment. This kind of yield is not common and should be emphasised. The ATX-specific activity is in line with the low picomolar per minute per milligram protein activity described in other reports. The tricks used for revealing these activity levels were to incubate large samples of biological materials with the substrate(s) for 6–14 h, using the appropriate controls. Because it was not clear if a single lpa-synthesis activity exists in the blood, some of the results should be interpreted with caution. The use of specific inhibitor, such as S32826 [36], seems to point to unique activity, with 100% inhibition in the presence of the compound. However, the possibility that this compound is also an inhibitor of another putative lpa-SA in biological media cannot be reasonably ruled out. A close examination of correlative data between ATX immunoreactivity and lpa production in patient blood samples strongly suggests that ATX is the only lpa-synthesising activity in blood [52]. A recent report [22] described ATX in blood, clarifying the roles of ATX and lpa. The most immediate information in respect to ATX in that study is the fact that, while circulating, ATX might very well be intimately associated with platelets. This fact would explain why we, and maybe others, had difficulties reproducing circulating ATX activity measurements beyond the choice of an adequate method, as discussed previously (see above). The manipulation of platelets, as they are very dynamic blood cells, is delicate, leading to variations in the amount of such cells in a given sample. Further careful assessments will be needed in the spirit of Nakamura's papers [53, 54] in order to assess a way of measuring ATX activity in blood, as this is directly linked to the key observations of Boucharaba et al. [55] concerning the metastasis process.

Finally, another perplexing fact is that, in blood from heterozygous $ATX^{-/+}$ mice, only 50% of ATX activity is present [22, 56]. From a pure enzymatic point of view, this observation is very surprising. At saturating substrate conditions, half the amount of enzyme incubated during 6–10 h should result in similar amount of product formed.

ATX in biological fluids

Autotaxin has been reported in most biological fluids: blister fluid [57], cerebro-spinal fluid [58, 59], plasma [35], peritoneal fluid [60], serum from various species (rabbit [16], bovine [17], human [53, 61]), egg white [40], urine [62] and synovial fluid [63].

ATX, being a main, or possibly sole (see above and [64] for further discussion), provider of extracellular lpa, is a major factor in many physiological features, and the presence of the enzyme in many of the biological fluids is not really surprising. Nevertheless, a major drawback is still under scrutiny. As pointed out by Nakamura et al.

[65], the measurement of lpa and lpc in biological fluids requires several precautions, without which these phospholipids are transformed (lpc into lpa by ATX and lpa into degradation metabolites) unless a series of precautions are taken. Furthermore, the stability of ATX in biological fluids is unknown, except for the α isoform (see above [11]), as well as its capacity to catalyse the lpc transformation in situ. Indeed, we and others have shown that the incubation of biological fluids (e.g. adipocyte conditioned media [18, 47]) led to massive production of lpa, supposedly from lpc, and the relevance of this observation in vivo is speculative.

Autotaxin and physio-pathology

Autotaxin has been identified as a marker of various types of diseases or pre-disease conditions, and it is expressed in a large number of tissues (see expression patterns in mice and man [11]). It seems that the role of ATX of providing connected tissues with massive amounts of lpa is key in many processes.

The ATX catalytic activity [66–68] is implicated in the early stages of embryo development. A minute description of the ATX expression pattern during mouse development has also been reported [67]. This feature might also explain why attempts to construct $ATX^{-/-}$ mice have failed.

The coordination between chondrocyte differentiation and joint formation by integrin during development depends on the expression of several proteins downstream of wnt, including ATX [69, 70]. Another publication described the expression of ATX in mesenchymal lineages, tracing the expression of ATX to cartilage/pre-cartilage condensations, joints, and so forth [71, 72]. On the contrary, ATX is down-regulated by IL-1β and IL-4 in the synoviocytes of rheumatoid arthritis patients [73]. Autotaxin has also been described in endothelial venules [74], and its expression was further induced by chronic inflammation [75]. The ATX secreted from epithelial cells induces the migration of intestinal cells [76].

Autotaxin has also been described as an obligatory passage of the basic fibroblast growth factor signalling machinery [77]; therefore, it could play a key role in angiogenesis, including related diseases such as cancer, macular degeneration, atherosclerosis and arthritis.

As stated previously, our initial interest was the possible link between the adipocyte production of ATX and adiposity, whether ATX is a positive or negative regulator of this process [18, 78]. Pre-adipocytes do not produce lpa or ATX, whereas adipocytes do [47]. When ATX was purified from adipocyte conditioned media [17], it was obvious that its role could be that of an autocrine regulator (positive or negative) of adipose tissue physiology [78]. These observations [18, 78] lead to the possible use of ATX as a marker of obesity/diabetes evolution and/or condition.

The ATX activity was also linked to neuropathic pain through its transformation of lpc into lpa in the spinal cord [79–81]. Furthermore, it has been suggested that ATX might also have a role in central nervous system diseases [82]. For example, studies have linked ATX and oligodendrocyte function [83, 84]. Concomitantly, the motility factor ATX has been described as playing a key role in myelination [83, 85, 86] and possibly in wound healing [57].

At several points, ATX seems to play a major role downstream of known oncogenes. It has been shown that ATX is induced by the over-expression and/or activation of some key oncogenes, such as ras [87, 88], jun [89–91], rhoC [92], myc (lpa regulated c-myc [93, 94], turning the system into a loop [95]), possibly fyn [96], and the proto-oncogene wnt-1 [69, 70] or integrins [97]. Song et al. [98] reported on the pathway(s) activated by ATX expression, and therefore its catalytic activity, presumably through the lpa receptor signalling cascade(s). Finally, ATX stimulates urokinase-type plasminogen activator through the nuclear factor κB signalling pathway [99] in human melanoma cells.

For instance, ATX has been described as being key in the metastatic process using expression profiles from many different cancer cells [100, 101], but also from primary cancer, such as thyroid carcinomas [102], ovarian cancer [103], and benign tumours such as uterine leiomyomas [104]. In prostate cancer, for example, ATX protein as well as ATX mRNA expression was compared between tumours and benign hyperplasia. It was shown that ATX is differentially expressed in non-cancerous (low) and cancerous (high) tissues, suggesting that ATX could be a marker of the process [105]. It has been suggested that serum ATX might be used as a marker in follicular lymphoma [61], uveal melanoma [106] or chronic hepatitis C [52, 107].

In summary, the presence of ATX might turn cancer cells into very aggressive, metastasis-induced tumours, with poor prognoses [108]. The metastasis process is complicated and has been reviewed in full by Steeg [109]. It should be pointed out that this review does not take into account lpa-mediated pathways, suggesting that the probable roles of ATX and lpa in metastasis are still poorly known.

Validation of autotaxin as a drug target

Quoting Kishi et al. [101]: “Enhanced ATX expression has been repeatedly demonstrated in various tumours, including non-small cell lung cancer, breast cancer, renal cell cancer, hepatocellular carcinoma and thyroid cancer

[102, 110–115]" and should be enough to suggest that ATX is a potential chemotherapeutic target. The most common ways to validate a target in pharmacology and, by extension, in pathological conditions is either genomically or by using pharmacological tools. Genomic validation includes population screening for SNPs or spontaneous mutations and micro-array experiments in vivo, ex vivo or in cellulo, constructing models in which the target has been deleted.

Pharmacological tools include the use of selective compounds, even if those compounds cannot be made into a drug. Concerning ATX, population genomic studies have not yet been reported in the literature, but micro-array experiments have been widely reported, though their interpretations for a given mRNA are not easy to access ex nihilo. In other words, ATX mRNA induction or repression might have been seen and reported during global studies, but the results might be buried in non-related publications, and, therefore, hard to reach. One should keep in mind that data coming from microarrays are often difficult to reproduce, essentially because of the numerous biological and technical parameters linked to this kind of analysis (see complete discussion in Ref. [116]). Several papers provide interesting information on ATX expression patterns. For instance, in human renal cell carcinomas, suppression subtractive hybridization experiments revealed a strong induction of ATX compared to normal, non-cancerous renal cells. Interestingly, a comparison with renal carcinoma cell lines showed that, in these isolated models (the cancer cell lines), ATX remained silent, or poorly expressed, suggesting again that regulation of ATX expression is dependent on upstream signals, possibly produced by the cellular environment of the tumour [113], a feature that was also considered in another model [55]. We took advantage of the availability of data from the NCI (http://discover.nci.nih.gov) that can be extracted and analysed for variations in ATX expression in a large panel of cancer cells. Figure 3 summarises the results. Some cells, but far from all, over-expressed ATX. It would be interesting to analyse the data through a correlative analysis as a possible marker of metastatic potential, as previously suggested. The amount of ATX mRNA in cancer cells has been profiled several times in a further exploration of the role of ATX in cancer development. Among those studies, a more in-depth investigation was conducted on the cloning of the promoter region [117] and its exploitation in order to understand the regulation of ATX in the cells, or in cancer in general [10]. Several links started to appear between the expression of ATX and the invasiveness of cancer cells, including lung cancer cells [110, 118], ras-transformed NIH-3T3 cells [13] and likely breast cancer-induced bone metastases [55]. In glioblastoma, an invasive malignant process, the ATX gene was among 21 genes over-expressed in invasive cells. Interestingly, these results were confirmed by immuno-staining [119].

Considering the second method of validating targets, three different groups have reported attempts to construct KO mice using different strategies [33, 56, 120]. In all cases, the deletion of the ATX gene or part of the gene, including our construct with mutated ATX at the catalytic site Thr^{210} (T210A mutation) [38], led to the compromised development of embryos at very early stages of in utero life. So far, no KO mice have been successfully created or, at least, reported in the literature.

Finally, pharmacological validation requires compounds as described in the following section.

Autotaxin inhibitors

Among the various approaches to finding inhibitors of ATX, the hypothesis (see Ref. [121] for review) that nature would provide control of this enzyme catalytic activity via small natural molecules that act as regulators has been explored by several groups: histidine was a candidate, rather than other amino acids [122], albumin [40] and the product(s) of the catalytic reactions [39] and cyclic phosphatidic acid (cPA) as naturally occurring compounds from the transphosphatidylation of lpc (see also [123] for reviews). The enzyme purified following this activity was ATX [124], suggesting a possible feedback loop regulating the activity of ATX in situ. Compounds derived from these observations and, therefore, from the natural substrates and products of the reactions have been synthesised (see [1] for review).

Several groups have reported on the search for potent inhibitors of ATX catalytic activity. This field has been covered by two recent reviews by Kano et al. [125] and Federico et al. [126], who pointed out the availability of lpa analogues that could be of great help in studying and validating the role of ATX in various pathologies. However, it turned out that these compounds are often difficult to use in vivo. These molecules, though, permitted the demonstration that ATX inhibition in several pathological conditions helps validate the target and its role, particularly in the context of metastasis. Of particular importance were the cyclic analogues of lpa, namely, cPA-stable analogues [127], which proved that the inhibition of ATX leads to a decrease in metastatic colonisation, a major breakthrough in the validation of ATX as a target in oncology.

One of the first compounds reported as a potent inhibitor of ATX activity was the immuno-modulator FTY720 (200 nM) [128], linking its previously described anti-cancer activity to this inhibiting activity. We initially reported a screen for ATX inhibitors in a small library of phosphodiesterase and kinase inhibitors [129] that identified new chemicals with mild inhibition potency towards ATX,

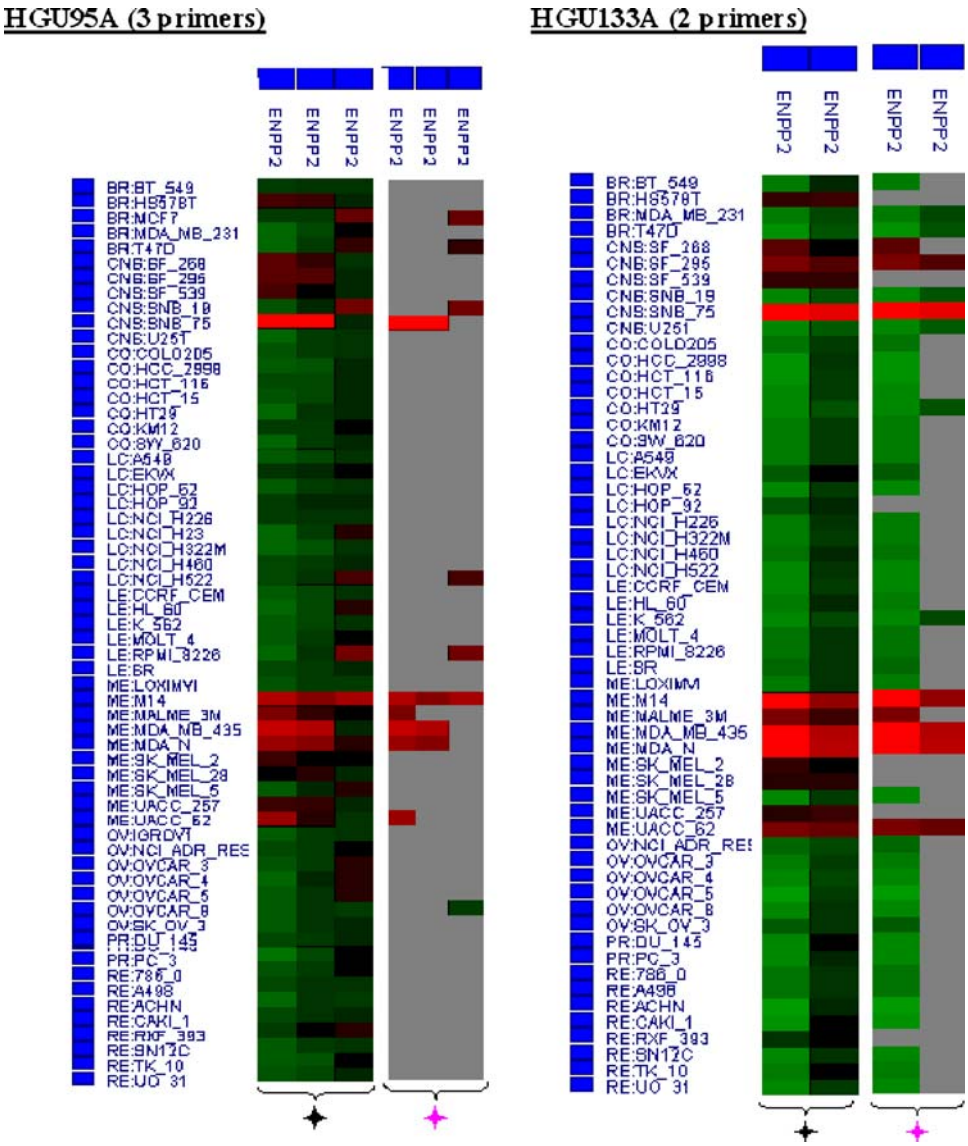

Fig. 3 Analysis of the expression of autotaxin (ATX) in 60 cancer cell lines from the ATCC using microarray analysis. cDNA microarrays were used to assess the gene expression profiles of 60 human cancer cell lines used in a drug discovery screen by the National Cancer Institute. Datasets are available online at http://discover.nci.nih.gov in the CellMiner program package [134]. HG-U95 and HG-U133A patterns have been integrated into our Resolver database (Rosetta, V7). The assessment of ATX status in these cell lines was achieved. *Red* Cell lines for which the ATX gene was strongly expressed compared to the mean value of the other cell lines (log ratio > 0). *Green* Cell lines for which the ATX gene was under-expressed compared to the mean value of the other cell lines (log ratio < 0). *Grey* Cell lines for which the ATX gene did not significantly vary (log ratio close to 0). *Black cross* Visualisation without a *p* value filter. *Pink cross* Visualisation of genes for which the expression did not vary significantly (p value ≤ 0.05 compared to the mean of the other cell lines)

including hypericin and damnacanthal, both micromolar ATX inhibitors. As expected, these compounds were already described for other pharmacological activities. Several laboratories have reported on the identification of small molecule inhibitors [130, 131], particularly our own; we performed a high-throughput screening campaign on a limited number of compounds ($\sim$20,000), leading to S32826, the most potent inhibitor reported thus far [36]. In that study, several features of this compound were described, including its biochemical properties, poor in vivo stability and its activity in several cellular models [36]. This compound also demonstrated, in cellular models, an impairment of the lysophosphatidate-induced protection of MCF-7 breast cancer and MDA-MB-435 melanoma cells against taxol-induced apoptosis [132], which is in line with previous observations of the protective role of lpa against apoptosis [133].

Perspectives

With a large number of papers published during the last few years, it is highly probable that inhibitors will be generated against ATX catalytic activity, opening new routes to therapeutic agents. As pointed out earlier, the current validation of the enzyme is not completely satisfactory, and further studies are needed to link beyond any doubt the activity of the enzyme, production of lpa and its role in the metastatic process.

Note added to proof A major paper to the validation of ATX as an essential target in metastasis was published at proof time: Liu et al., Cancer Cell, 2009, 15:539–550.

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