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Controlling cancer through the autotaxin–lysophosphatidic acid receptor axis

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Abstract

LPA (lysophosphatidic acid, 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate), is a growth factor-like lipid mediator that regulates many cellular functions, many of which are unique to malignantly transformed cells. The simple chemical structure of LPA and its profound effects in cancer cells has attracted the attention of the cancer therapeutics field and drives the development of therapeutics based on the LPA scaffold. In biological fluids, LPA is generated by ATX (autotaxin), a lysophospholipase D that cleaves the choline/serine headgroup from lysophosphatidylcholine and lysophosphatidylserine to generate LPA. In the present article, we review some of the key findings that make the ATX–LPA signalling axis an emerging target for cancer therapy.

Keywords

autotaxin; cancer; drug discovery; lysophosphatidic acid (LPA); 4-pentadecylbenzylphosphonic acid (4-PBA); therapy

The many roles of autotaxin and lysophosphatidic acid in malignancy

ATX (autotaxin) is a member of the NPP (nucleotide pyrophosphatase/phosphodiesterase) enzyme family and is also known as NPP2 [1,2]. ATX was originally identified as a secreted phosphatase in melanoma culture supernatant that promoted cancer cell motility [3]. Ten years later, ATX was identified as a lysophospholipase D responsible for the production of

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LPA (lysophosphatidic acid, 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate) in blood [4,5]. ATX-KO (knockout) mice die *in utero* due to angiogenic defects, whereas heterozygous ATX-KOs appear to be normal, but have half the plasma LPA concentration compared with wild-type littermates [6,7].

Many cancers secrete ATX, which contributes to their invasive properties (Figure 1). Ovarian cancer cells produce high levels of LPA [8,9]. Whether plasma LPA levels due to ATX production by cancer cells are elevated and can be used as a cancer biomarker has generated some controversy. The plasma ATX level does not appear to be a cancer marker because it is also elevated in patients with liver disease [10,11], during pregnancy [12] and in patients with acute coronary syndrome [13]. Plasma LPA levels follow the trend of plasma ATX levels. Gene copy numbers are elevated in ovarian cancers in chromosomal region 8q24, which contains the gene encoding ATX [14]. There is consensus in the field that LPA levels are highly elevated in ovarian cancer ascites [8,15], but also in tumour cell effusates from other types of malignancies [8]. The acyl chain composition of plasma LPA and the LPA in tumour cell effusates differ suggesting that LPA does not freely cross the barriers between body compartments [8]. Plasma LPA level has been found elevated in only a select group of malignancies that include pancreatic cancer [16] and follicular lymphoma [17]. Extreme care must be exercised during the collection, handling and storage of blood samples intended for ATX and LPA analysis because LPA production continues *ex vivo* due in part to the removal of the LPA degradation by cells of the vasculature and the liver, and also due to the continued action of ATX [18] and the release of phospholipases A_1 and A_2 from platelets [19].

ATX is among the 40 most up-regulated genes in highly metastatic cancers [20]. Ectopic expression of ATX in mice leads to mammary intraepithelial neoplasia, developing into invasive and metastatic tumours [21]. ATX inhibits paclitaxel-induced apoptosis in breast cancer cells [22], and we have shown that LPA renders ovarian cancer cells chemoresistant to cisplatin and adriamycin [23]. ATX becomes overexpressed in patients with recurrent disease after treatment with chemotherapy [24]. In a genome-wide siRNA (small interfering RNA) screen, we identified ATX as a candidate drug-resistance gene in ovarian cancer and showed that an inhibitor of ATX increases paclitaxel sensitivity of resistant cancer cells [25].

LPA receptors in cancer cells

LPA activates two clusters of GPCRs (G-protein-coupled receptors). The EDG (endothelial differentiation gene) cluster includes the LPA_1, LPA_2 and LPA_3 receptors that are close relatives of the EDG family sphingosine 1-phosphate receptors. LPA_1 and LPA_2 are broadly expressed. Many cancers overexpress multiple subtypes of LPA receptors [26]. LPA_1 has been shown to be a regulator of cancer cell motility and metastasis [27] and matrix metalloproteinase expression [28]. LPA_2 plays an important role in the invasiveness of ovarian cancer in at least two ways. First, LPA_2 promotes production of VEGF (vascular endothelial growth factor), uPA (urokinase-type plasminogen activator) and matrix metalloproteinases [29]. LPA increases VEGF production, and in turn up-regulates ATX production, which increases LPA levels [30], another potential feedforward loop that also promotes angiogenesis. Secondly, LPA-induced uPA production promotes invasiveness of ovarian cancer cell lines [31]. Clinically, high uPA levels indicate poor prognosis in ovarian cancer patients [32].

A second cluster of LPA receptors is found within the purinergic P2Y gene cluster. The better characterized members of this cluster are LPA_4 (P2Y9), LPA_5 (GPR92) and LPA_6 (P2Y5). Mouse embryonic fibroblasts isolated from LPA_4-KOs showed increased migration

to LPA and developed spontaneous tumours [33], suggesting that LPA_4 has a tumour-suppressor role. There are additional related GPCRs that appear to be activated by LPA. In chronological order of discovery, these include GPR87, P2Y10 and GPR35, but their biological function remains to be characterized.

Thus compelling evidence exists that the overexpressed LPA receptors play a fundamental role in carcinogenesis, invasiveness, metastatic potential and therapeutic resistance in many types of carcinomas.

Juxtacrine signalling between the metastatic cancer cell and cells of the endothelial or mesothelial barrier

ATX via LPA production promotes metastasis. During haematogenous metastasis, cancer cells have to cross the vessel wall twice, first when entering the blood stream and secondly when transmigrating it to seed the site of metastasis. For this reason, the role of LPA and ATX in the interaction between the cancer cell and the vascular endothelial cell is of peculiar importance. ATX–LPA signalling takes place in the cancer cell microenvironment (Figure 1). The recent elucidation of the crystal structure of ATX [34,35] reinforces this concept in two ways: first, the interaction of the ATX somatomedin B domain with β3 integrin provides the structural framework for localizing ATX-mediated LPA production to the cancer and endothelial cell surfaces, which express β3 integrin. Secondly, identification of an LPA-binding site within a hydrophobic tunnel of ATX important for releasing LPA at the cell surface juxtaposed to the LPA receptor provides yet another structural framework for juxtacrine signalling. Thus dual targeting of ATX and LPA receptors for the control of metastasis and tumour growth is a logical extension of the juxtacrine signalling concept because one inhibits both targets that are spatially and functionally linked in the cancer cell microenvironment.

Validation of ATX as a drug target for metastasis control

We examined the effect of silencing ATX expression in the B16 melanoma metastasis model syngeneic to the C57BL/6 mouse strain. ATX expression was knocked down using a lentivirally delivered shRNA (small hairpin RNA) construct (ShATX, Figure 2). ATX-KD (knockdown) reduced the expression of ATX mRNA and protein (Figures 2A and 2B) and also reduced the lysophospholipase D activity secreted into the conditioned medium compared with wild-type B16 or Scr (scrambled) shRNA-transduced cells (Figure 2C). We examined the effect of ATX-KD using a transcellular migration assay described previously by Mukai et al. [36]. Briefly, ShATX and Scr shRNA transduced cells (10^5 cells/ml) were seeded on to a confluent rat mesothelial cell monolayer and increasing concentrations of LPC (lysophosphatidylcholine) or 3 μM LPA were added. After 6 h of incubation, the medium was removed and the monolayer was washed and fixed. The number of B16 melanoma cells that penetrated the mesothelial monolayer was counted under a phase-contrast microscope. LPA treatment increased the transmonolayer invasion of the ShATX and control cells (Figure 2D). Addition of the ATX substrate LPC to Scr shRNA-transduced melanoma cells dose-dependently increased invasion. In contrast, LPC failed to induce invasion of the ShATX-transduced melanoma cells. These observations support the hypothesis that down-regulation of ATX activity in the tumour cells alters their invasive capacity.

To extend these *in vitro* observations, we injected 5×10^5 Scr- and ShATX-transduced B16 melanoma cells into C57BL/6 mice via the tail vein. At 3 weeks post-inoculation, animals were killed, the lungs were washed, inflated and fixed with formalin, and the number of lung metastases was determined. Melanoma cells with ATX-KD showed a 50% reduction in the

number of metastasis compared with control cells (Figure 2E). This observation reinforces our *in vitro* findings and supports a role for ATX in metastasis.

Drug discovery targeting ATX

On the basis of the relevance of the ATX–LPA–LPA receptor signalling axis to many types of cancers, it is reasonable to propose drug discovery aimed at ATX and LPA receptors, or the combination of these targets to prevent and inhibit cancer invasion, metastasis and growth. At present, there are no FDA (U.S. Food and Drug Administration)-approved drugs to either of these targets. ATX is continuously turning over with a half-life estimated to be less than 1 h [37]. The half-life of LPA injected into blood is of the order of a few minutes, indicating that this pool of LPA is rapidly turning over [38]. These two properties taken together suggest that ATX has to be inhibited for a long duration in order to significantly affect LPA production. We envisage that the drug candidates targeting ATX and/or LPA receptors will ultimately be used as part of the multimodal treatment of cancers for the prevention of metastasis before or after removal of the primary tumour. We must not ignore the fact that ATX inhibitors have therapeutic utility in controlling inflammatory diseases as well, because of the documented role of ATX in autoimmunity [39], asthma [40], macular degeneration [41] and neuropathic pain [42].

We reported recently the characterization of a new ATX inhibitor 4PBPA (4-pentadecylbenzylphosphonic acid) [43]. 4PBPA has a relatively long (10.5 h) half-life and it reduced plasma LPA levels by 50% over a 24-h period [43]. This inhibitor was tested on the invasion of confluent monolayers of HUVECs (human umbilical vein endothelial cells) by MM1 hepatoma cells in 1% low-serum medium with or without 5 μM LPC (Figure 3A). MM1 cells express ATX, whereas the mesothelium and HUVECs show very low expression, and we were unable to detect ATX activity in HUVEC-conditioned medium [43]. Thus the source of ATX in this system is primarily from the MM1 carcinoma cells. Addition of LPC increased invasion significantly, whereas 4PBPA dose-dependently blocked the LPC-induced invasion of MM1 cells on the HUVEC monolayers. These types of monolayer invasion assays have been considered as excellent *in vitro* models for cancer invasion because the invading cells must penetrate and migrate below the monolayer, resembling the process that takes place during metastasis [36]. We also examined the effect of 4PBPA or vehicle using the B16 melanoma metastasis model *in vivo*. Daily injections starting 30 min after melanoma inoculation were continued until day 20. On day 21, mice were killed, the lungs were harvested, and the number of melanoma nodules in the lungs was counted. In this treatment paradigm, 4PBPA reduced the number of metastases from an average of 42 per mouse in the vehicle group to eight per mouse (Figure 3B).

To examine the role of ATX inhibition on the micro-to-macro metastasis transition, we performed the same experiment by delaying the first treatment with 4PBPA from 30 min to 96-h post-inoculation. The treatment continued with single daily doses of 4PBPA until day 20. The mice were killed on day 21 and the lung metastases were counted. 4PBPA injection starting up to 48 h post-inocculation caused a highly significant reduction in the number of metastases. However, when the treatment started at 72 h post-inocculation, the reduction of metastases, although significant, began to diminish and when treatment began on 96 h after inoculation it was no longer effective (Figure 3B). These results point to an important limitation of ATX inhibition therapy and indicate that blocking of ATX activity only reduces the seeding of metastases, but does not inhibit tumour growth. These results with the melanoma model corroborate similar findings obtained in a breast cancer metastasis model by the Peyruchaud group [44].

Dual targeting of ATX and LPA receptors controls metastasis and tumour growth

LPA exerts feedback inhibition on ATX [45]. This observation leads to the concept that one should be able to find LPA analogues that inhibit ATX without activating any of the LPA receptor subtypes. We obtained proof of this principle through the development of carba-cyclic phosphatidic acid analogues that inhibited ATX without the activation of $LPA_{1–4}$. Carba-cyclic phosphatidic acid inhibited MM1 carcinoma and A2058 melanoma invasion *in vitro* and also reduced the number of B16 melanoma metastasis in mice [46–48]. In collaboration with the Prestwich group, we have characterized several lipid analogues of LPA that inhibit ATX and also function as antagonists of multiple LPA receptors [49]. One of the LPA bromophosphonate enantiomers was highly effective in blocking the metastasis and growth of xenotransplanted human breast, colon and liver carcinomas in nude mice [49,50].

The studies outlined in the present review provide the foundation for drug discovery targeting the ATX–LPA–LPA receptor signalling axis. The lack of phenotype in heterozygous ATX-KO mice with 50% lower plasma LPA levels and the initial data using ATX inhibitors over extended periods of time for the treatment of tumour-bearing mice revealed no obvious side effects. There are numerous publications that have appeared in the literature during the last 2 years describing multiple ATX inhibitors. The race is on now to find the one that has ideal pharmacokinetics and bioavailability with minimal side effects. We predict that, within the next few years, many new chemical scaffolds that block ATX and LPA receptors will be identified. Today, at the dawn of personalized medicine, we envisage that combination treatment protocols consisting of a long-acting ATX inhibitor and individual LPA receptor antagonists matching the expression profile of the patient's malignancy will emerge as valuable modalities of cancer treatment and metastasis control.

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Abbreviations used

ATX	autotaxin
EDG	endothelial differentiation gene
GPCR	G-protein-coupled receptor
HUVEC	human umbilical vein endothelial cell
KD	knockdown
KO	knockout
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
NPP	nucleotide pyrophosphatase/phosphodiesterase
4PBA	4-pentadecylbenzylphosphonic acid
Scr	scrambled

shRNA	small hairpin RNA
siRNA	small interfering RNA
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor

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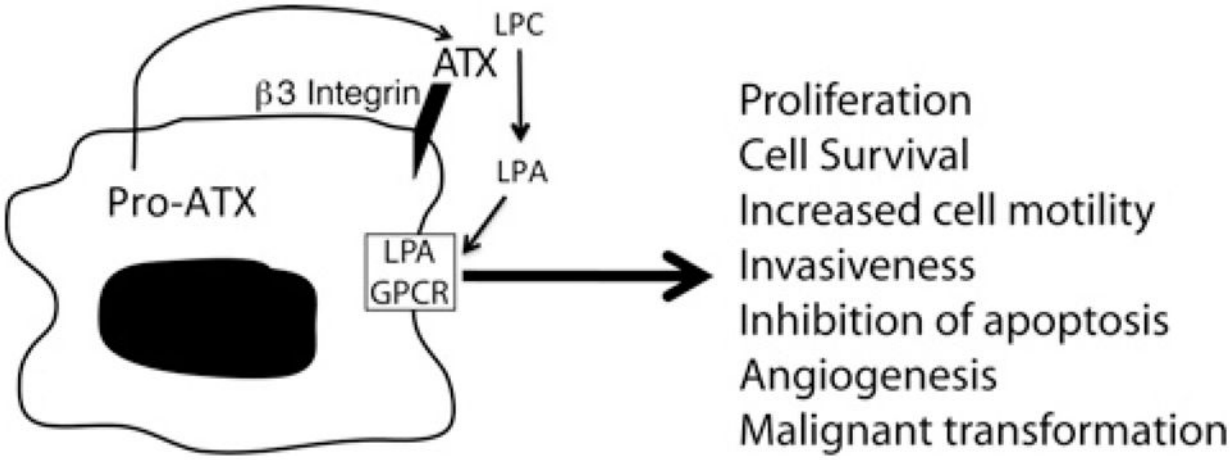

Figure 1. Juxtacrine signalling via the ATX–LPA receptor axis

ATX secreted from the tumour cell can be captured at the cell surface through binding to β3 integrin in the vicinity of the LPA GPCR. Activation of the LPA GPCR elicits responses that modulate the biological properties of the cancer cell.

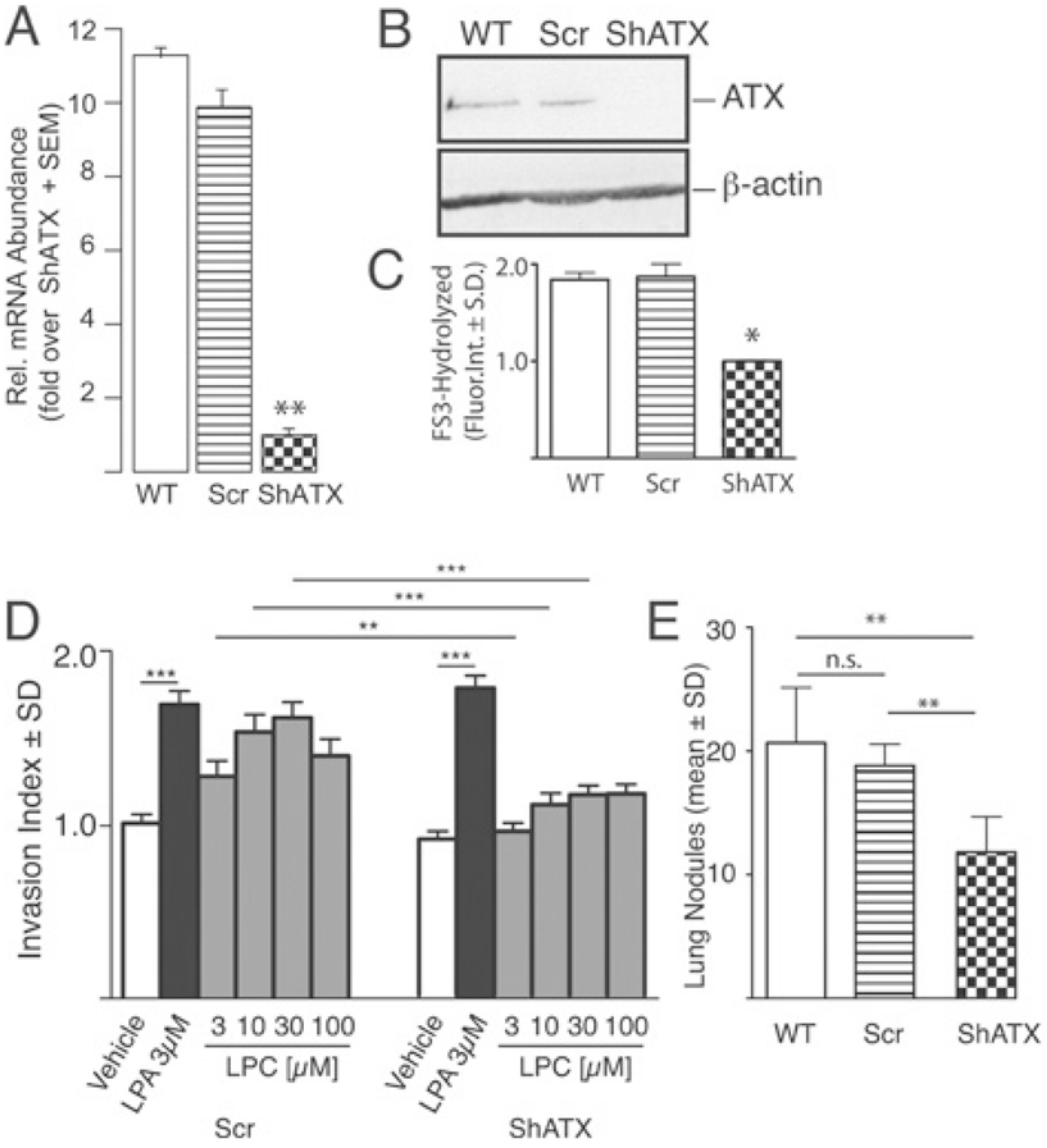

Figure 2. Down-regulation of ATX in B16-F10 melanoma cells inhibits invasion *in vitro* and metastasis *in vivo*

B16 cells were transduced with lentivirus encoding a shRNA to ATX (ShATX) or an Scr construct. mRNA level (**A**), ATX protein expression (**B**) and ATX catalytic activity in 48-h conditioned medium measured using FS-3 substrate (**C**) were all down-regulated in ShATX cells compared with Scr and wild-type (WT) B16-F10 cells. Scr cells showed an LPC dose-dependent increase in invasion of rat mesothelial monolayers, whereas ShATX cells failed to respond to LPC (**D**). Both cell types responded equally to LPA with increased invasion of the monolayer. When injected into C57BL/6 mice (5×10^4/cell per mouse in 100 μl) via the tail vein, ShATX cells yielded 50% fewer metastases compared with Scr and WT B16-F10 melanoma cells (**E**). n.s., not significant; *$P < 0.05$; **$P < 0.01$; $P < 0.001$. Reproduced from [43] Gupte, R., Patil, R., Liu, J., Wang, Y., Lee, S.C., Fujiwara, Y., Fells, J., Bolen, A.L., Emmons-Thompson, K., Yates, C.R., Siddam, A., Panupinthu, N., Pham, T.C., Baker, D.L., Parrill, A.L., Mills, G.B., Tigyi, G. and Miller, D.D. (2011) Benzyl and naphthalene methylphosphonic acid inhibitors of autotaxin with anti-invasive and anti-metastatic

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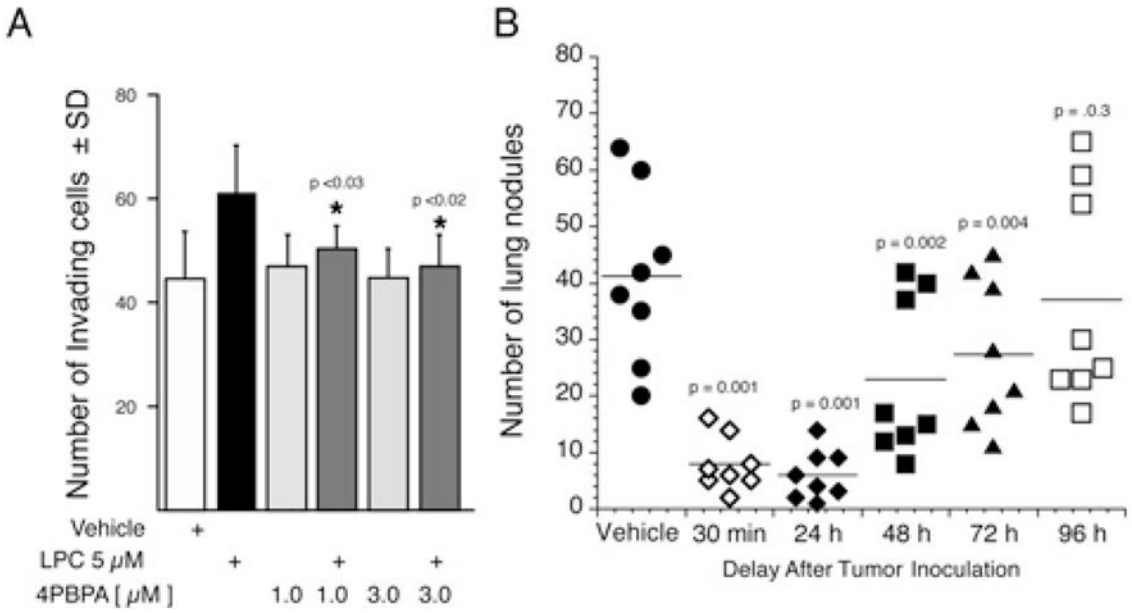

Figure 3. Pharmacological inhibition of ATX blocks tumour cell invasion *in vitro* and seeding of metastasis *in vivo*

The benzyl-methyl phosphonate ATX inhibitor 4PBPA inhibits the invasion of HUVEC monolayers by MM1 rat hepatocarcinoma cells induced by 5 μM LPC added to the medium (**A**). 4PBPA does not inhibit tumour growth, but reduces the number of metastases when administered up to 72 h post-inocculation of the tumour cells. 4PBPA (1 mg/kg) was injected daily starting at different times after tumour inoculation (7.5×10^4 cells/mouse) and continued up to day 20. The number of lung metastases was counted on day 21. Note that 4PBPA yields very effective protection against the establishment of micrometastases even when administration began at 48 h after inoculation of B16-F10 melanoma cells via the tail vein. Reproduced from [43] Gupte, R., Patil, R., Liu, J., Wang, Y., Lee, S.C., Fujiwara, Y., Fells, J., Bolen, A.L., Emmons-Thompson, K., Yates, C.R., Siddam, A., Panupinthu, N., Pham, T.C., Baker, D.L., Parrill, A.L., Mills, G.B., Tigyi, G. and Miller, D.D. (2011) Benzyl and naphthalene methylphosphonic acid inhibitors of autotaxin with anti-invasive and anti-metastatic activity. ChemMedChem **6**, 922–935. Copyright Wiley-VCH Verlag GmbH & Co. KGaA with permission.