Identification of small-molecule inhibitors of autotaxin that inhibit melanoma cell migration and invasion

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Abstract

Autotaxin (ATX) is a prometastatic enzyme initially isolated from the conditioned medium of human melanoma cells that stimulates a myriad of biological activities, including angiogenesis and the promotion of cell growth, survival, and differentiation through the production of lysophosphatidic acid (LPA). ATX increases the aggressiveness and invasiveness of transformed cells, and ATX levels directly correlate with tumor stage and grade in several human malignancies. To study the role of ATX in the pathogenesis of malignant melanoma, we developed antibodies and small-molecule inhibitors against recombinant human protein. Immunohistochemistry of paraffin-embedded human tissue shows that ATX levels are markedly increased in human primary and metastatic melanoma relative to benign nevi. Chemical screens identified several small-molecule inhibitors with binding constants ranging from nanomolar to low micromolar. Cell migration and invasion assays with melanoma cell lines show that ATX markedly stimulates melanoma cell migration and invasion, an effect suppressed by ATX inhibitors. The migratory phenotype can be rescued by the addition of the enzymatic product of ATX, LPA, confirming that the observed inhibition is linked to suppression of LPA production by ATX. Chemical analogues of the inhibitors show structure-activity relationships important for ATX inhibition and indicate pathways for their optimization. These studies suggest that ATX is an approachable molecular target for the rational design of chemotherapeutic agents directed against malignant melanoma.

Introduction

Autotaxin (ATX) is a secreted glycoprotein member of the nucleotide pyrophosphatase/phosphodiesterase family of enzymes that was first identified as a motility-stimulating factor in melanoma cells (1). ATX has both the phosphodiesterase (PDE) activity expected of nucleotide pyrophosphatase/phosphodiesterases (2, 3) and also a lysophospholipase D (lysoPLD) activity unique among this family (4–7). Both PDE and lysoPLD activities occur at the same ATX active site, although PDE activity is considerably weaker and is unlikely to have physiologic relevance (2, 4, 8).

The lysoPLD activity of ATX generates lysophosphatidic acid (LPA) from lysophosphatidylcholine (4, 5, 9), although it can hydrolyze other lysolipids as well, such as sphingosylphosphorylcholine (9). ATX is the sole source of extracellular LPA, as shown by transgenic animal experiments in which heterozygous ATX knockout mice possess half the ATX activity and serum LPA levels observed in their wild-type counterparts (10). LPA mediates a broad range of biological activities through the activation of G protein–coupled cell surface receptors to stimulate events central to organismal fate, such as wound healing, brain development, and vascular remodeling (11).

Although ATX is not responsible for oncogenic transformation, it has been shown to increase tumor invasiveness, metastasis, and neovascularization (12, 13). In addition, recent studies of ATX knockout mice suggest that ATX contributes to tumor progression by stabilizing blood vessels in the vicinity of tumors (14). The potent mitogenic activity of human ovarian cancer ascitic fluid is mediated by LPA and linked to ATX activity, and ATX is up-regulated in tumor cells at the leading edge of the locally invasive human brain tumor glioblastoma multiforme (15). ATX is increased in Hodgkin’s lymphoma cells, which carry the EBV, and is thought to mediate an aggressive phenotype in EBV-positive Hodgkin's lymphoma (16). In addition, LPA signaling plays a role in the motility and metastasis of prostate cancer (17, 18), suggesting a role for ATX in prostatic adenocarcinoma.
The increased expression of ATX in a wide variety of human tumors relative to normal tissues has been established by multiple complementary techniques, including the quantification of mRNA levels by in situ hybridization and quantitative PCR, and the quantification of protein levels by immunohistochemistry and Western blotting. ATX was first cultured from the conditioned medium of human melanoma cells (1), and three of four (75%) melanoma cell lines tested were reported to over-express and secrete ATX (including cell line A2058; ref. 19). Other tumors with increased ATX expression include breast cancer (where it correlates with tumor invasiveness; ref. 20), teratocarcinoma (3), neuroblastoma (where it correlates with the more aggressive and lethal variant commonly observed in older patients; ref. 21), glioblastoma (where expression is greater in the leading edge of invasive tumor cells compared with the tumor core; refs. 15, 22), lung carcinoma [where overexpression is found in 7 of 12 (58%) tumor cell lines; ref. 23], thyroid carcinoma (where it correlates with the aggressive anaplastic variant of thyroid carcinoma compared with the less aggressive follicular thyroid carcinoma cell lines; ref. 24), and ovarian cancer (where anatomical levels of the enzymatic product of ATX are found in the malignant ascitic fluids; refs. 25–28). Taken together, the data indicate that ATX functions as a tumor motility and angiogenic factor, stimulating multiple facets of the metastatic cascade to promote aggressive variants of human malignancies.

Primary malignant melanoma usually presents with cutaneous lesions that can be readily treated surgically, but metastatic melanoma is poorly controlled surgically and chemotherapeutically and often follows an ominous clinical course. ATX is an extracellular prometastatic enzyme and therefore an attractive molecular target for melanoma because inhibitory compounds can reach the target site without having to cross the cell membrane. LPA analogues are effective ATX inhibitors (29–32) and successfully inhibit tumor growth in animal models (33), but LPA mimics could also bind and activate LPA receptors initiating the signaling cascades an ATX inhibitor is intended to stop. Two recent studies identified several small-molecule ATX inhibitors (32, 33). However, the binding constants of the inhibitors were not measured nor were the effects on cancer cell migration.

In this study, we establish ATX as a molecular target to inhibit malignant melanoma metastasis by verifying over-expression in primary and metastatic melanoma, identifying small-molecule ATX inhibitors, and quantifying their effects on melanoma cell motility and invasion. We identify four inhibitors of ATX with inhibitory constants in the nanomolar or low micromolar range. We further validate the inhibitors using in vitro cell motility (Boyden chamber) and cell invasion (Matrigel) assays on human melanoma cell lines. Structural analogues of a subset of the inhibitors reveal chemical moieties responsible for inhibition. A chemical scaffold with high affinity (nmol/L) to ATX is identified, as well as an inhibitor with low in vivo toxicity, as it is orally bioavailable and has undergone Food and Drug Administration approval. These studies establish a rational path for the development of antimetastatic compounds, which may be useful in elucidating the pathogenesis of melanoma spread and metastasis and may point to the rational design of future chemotherapeutic agents in the treatment of melanoma.

Materials and Methods

Reagents

All chemicals and reagents were the highest purity commercially available. 3-Nitrophenyl 5′-thymidine monophosphate (pNPM) was purchased as a dry powder from Sigma. Fluorescent Substrate-3 (FS-3) came from Echelon. Both substrates were freshly dissolved in assay buffer [50 mmol/L Tris, 5 mmol/L KCl, 140 mmol/L NaCl, 1 mmol/L MgCl2, 1 mmol/L CaCl2 (pH 8.0)] immediately before use. RJC 03297 was purchased from Maybridge, and NSC 48300 was obtained from the National Cancer Institute Developmental Therapeutics Program Open Chemical Repository. The structure and purity (95%) of NSC 48300 was confirmed by 1H nuclear magnetic resonance and mass spectroscopy. Analogues of NSC 48300 were identified by doing a substructure search against the National Cancer Institute Open Chemical Repository collection. Analogues deemed useful for establishing structure-activity relationships were identified and individually requested from the Developmental Therapeutics Program. Hexachlorophene, merbromin, bithionol, 2,2′-methylenebis(4-chlorophenol), and eosin Y were purchased from Sigma and all inhibitors were solubilized at 10 to 100 mmol/L in DMSO for steady-state kinetics and DMSO [hexachlorophene, bithionol, 2,2′-methylenebis(4-chlorophenol), and NSC 48300] or water (merbromin) for cell motility assays.

Cell Culture

Human melanoma cells A2058 (American Type Culture Collection) were grown in DMEM (Life Technologies); Mel 28 (American Type Culture Collection), YUSAC2 (Yale University), and Mel-888 (Yale University) were grown in McCoy’s 5A medium (American Type Culture Collection). Human breast adenocarcinoma cells MDA-MB-231 (American Type Culture Collection) and ovarian carcinoma cells R182 (Gil Mor, Yale University) were grown in RPMI 1640 (Life Technologies). All cell media were supplemented with 10% (v/v) FCS (Life Technologies) and 1% (v/v) 1-glutamine (Life Technologies) and grown at 37°C in a humidified atmosphere containing 5% CO2.

Protein Expression and Purification

The full-length human NPP2 gene (National Center for Biotechnology Information accession BC034961) followed by a COOH-terminal TEV cleavage site and a 9-His and
6-His purification tag was cloned and transferred into a p-DEST-8 baculovirus shuttle vector (Invitrogen), and bacmid DNA was generated by standard methods. The DNA sequence and correct transposition of the genes was verified by PCR, and recombinant full-length human ATX was expressed in High Five insect cells using standard methods (34). Three days after infection, the supernatant was adjusted to a final concentration of 50 mmol/L Tris (pH 8.0), 5 mmol/L CaCl₂, and 1 mmol/L NiSO₄ and stirred for 10 min at room temperature. The resulting precipitant was removed by centrifugation (4,420 × g for 30 min) and filtration (0.45 µm cutoff), and the supernatant was concentrated using a Pall concentration system to ~250 mL.

The cell medium was passed over a nickel affinity column (5 mL, FastFlow resin), equilibrated in binding buffer [20 mmol/L Tris (pH 8.0), 300 mmol/L NaCl, 20 mmol/L imidazole, 20% ethylene glycol] at 4°C, washed with 10 column volumes of binding buffer, and then eluted with binding buffer supplemented with 300 mmol/L imidazole. Purified ATX was concentrated to ~10 mg mL⁻¹ and dialyzed into the appropriate assay buffer.

**Polyclonal Antibody Production and Staining**

Polyclonal antibodies to recombinant human ATX were generated in chickens by ProSci, Inc. Antibodies were purified with Eggcellent Chicken IgY Purification kit (Pierce). Tissue microarrays containing 10 examples each of normal skin, nevi, primary melanoma, and metastatic melanoma were obtained from the Yale Tissue bank, and the slides were stained using a 1:1,000 dilution of purified antibody.

**Inhibitor Screen**

High-throughput screens were done against two libraries: the GenPlus library of 960 compounds (NINDS Custom Collection, MicroSource Discovery Systems) and the National Cancer Institute Diversity Set (1990 compounds).6 The screens were done at the Yale Center for Chemical Genomics using a Tecan Aquarius robot in combination with an Aquarius liquid handler and a Freedom EVO Workstation. A V&P Scientific 384-pin tool transferred small molecules (10 mmol/L stock) from library plates into 384-well assay plates, and an Aquarius robot was used to plate 20 µL of assay buffer [50 mmol/L Tris, 140 mmol/L NaCl, 1 mmol/L MgCl₂, 5 mmol/L CaCl₂ (pH 8.0)], 1.25 µL of sample, and 0.25 µL of antibody into the optically clear, 384-well assay plates to a total volume of 150 µL. The assays were done in a total volume of 50 µL.

**Steady-State Enzyme Assays**

PDE activity of ATX (75–100 nmol/L) was measured by absorbance (λ = 405 nm) on a SpectraMax 250 plate reader at 25°C with the substrate pNP-TMP (36). Reactions were run in assay buffer containing 10% DMSO (v/v) and 1 mg mL⁻¹ bovine serum albumin (hexachlorophene, merbromin, and bithionol) or without bovine serum albumin [NSC 48300, 2,2'-methylenebis(4-chlorophenol), eosin Y, and RJC 03297] in a total volume of 150 µL. The uncatalyzed reaction rate was ≤10⁻⁷ s⁻¹ (data not shown).

LysoPLD activity of ATX (75–225 nmol/L) was measured using the fluorescent lysophosphatidylcholine analogue FS-3 (37). Fluorescence was measured (λ ex = 485 nm; λ em = 520 nm) on a SpectraMax Gemini XPS at 37°C under buffer conditions similar to the PDE assays but lacking bovine serum albumin. Fluorescence intensity was corrected for inner filter effects (38) using fluorescein. The spontaneous reaction rates were subtracted from the enzyme catalyzed rates.

The steady-state rates (v) of product formation were obtained by fitting the time courses of absorbance or fluorescence change to linear functions. Absorbance units were converted to p-nitrophenolate concentration using ε₄₀₅ of 18.5 mmol/L cm⁻¹ (39) and a measured path length of 0.494 cm. FS-3 hydrolysis product formation was quantitated in arbitrary fluorescence units.

The Michaelis constants (K M) and the catalytic turnover rate constants (k cat) were obtained by fitting the [substrate] dependence of v to a rectangular hyperbola in the form of the Briggs-Haldane equation:

\[
\frac{v}{[E]_{tot}} = \frac{k_{cat}[S]}{K_M + [S]} \\
(\text{Eq. A})
\]

In which [S] is [pNP-TMP] or [FS-3]. Inhibitors were treated as reversible inhibitors and analyzed according to the general steady-state linear enzyme inhibitor scheme:

\[
I + E + S \overset{k_i}{\leftrightarrow} EI \overset{k_{a}}{\rightarrow} ES \overset{k_{cat}}{\rightarrow} E + P
\]

where E is ATX, S is pNP-TMP or FS-3 substrate, P are the hydrolysis products, and K i is the dissociation constant for inhibitor binding. The values of k a and K i were obtained by fitting the steady-state rates of product formation (v) to the general inhibition equation:

\[
\frac{v}{[E]_{tot}} = \frac{k_{cat}[S]}{K_S \left(1 + \frac{[I]}{K_i} \right) + [S] \left(1 + \frac{[I]}{k_a} \right)} \\
(\text{Eq. B})
\]
in which $[I]$ is the inhibitor concentration, $k_{cat}$ is the maximum catalytic turnover rate, and $K_S$ is the Michaelis constant of the substrate obtained in the absence of inhibitor.

The type of inhibition (competitive, noncompetitive, or uncompetitive) depends on the value of $\alpha$. Competitive inhibitors have $\alpha > 1$ and thus fit the equation:

$$
\frac{v}{[E]_{tot}} = \frac{k_{cat} [S]}{K_S (1 + \frac{[I]}{K_I}) + [pNP - TMP]} \quad \text{(Eq. C)}
$$

Noncompetitive inhibition is the special case in which $\alpha = 1$, which fits the equation:

$$
\frac{v}{[E]_{tot}} = \frac{k_{cat} [S]}{(K_S + [pNP - TMP]) (1 + \frac{[I]}{K_I})} \quad \text{(Eq. D)}
$$

Mixed inhibition occurs when $\alpha$ differs slightly (<10-fold) from unity so that a single inhibition type does not dominate the effect and the general inhibition equation is used. Uncompetitive inhibition was not observed in this study. The type of inhibition was assigned if the difference between the $r^2$ values of the fits to the general inhibition equation (Eq. B) and the specific inhibition (i.e., competitive, noncompetitive, or uncompetitive) equations was <0.01.

Cell Motility Assays

Microchemotaxis chambers were used in Boyden chamber cell migration assays on a human melanoma cell line (A2058) known to display LPA-dependent chemotaxis (40). The assay chambers are divided by a gelatin-coated membrane with 8-μm/L pores (NeuroProbe). Chemoattractant (ATX inhibitor) and/or controls were placed in the lower half of the chamber, and the cells were placed in the upper chamber. The chambers were then incubated at 37°C for 4 h, after which the filters were removed, fixed in 100% methanol, stained with a DiffQuick solution, and mounted onto glass slides. Nonmigrating cells were removed from the membrane by scraping the top surface. The cells were quantitated by counting the cell number per high-power field in the various wells. Treatments were compared using a $\chi^2$ test.

Cell Invasion Assays

Matrigel matrix (1 mg/mL; Becton Dickinson) was added to 8-μm pore gelatin-coated membranes placed in cell culture inserts (BD Falcon) and allowed to set for 24 h at room temperature. The inserts were placed into companion 24-well plates (BD Falcon), and chemoattractants (ATX ± inhibitors) and/or controls were added to the bottom of the wells. Cells (1 × 10⁶) were added to the inserts and incubated for 3 h at 37°C. Following incubation, the inserts were removed, fixed in 100% methanol, stained with DiffQuick solution, and mounted on glass slides. Non-invasive cells were removed by scraping away the Matrigel, and migrating cells were quantitated by counting cell number per high-power field.

Results

Purification of ATX

Purified ATX migrates as a single band of ~100 kDa by SDS-PAGE. The ATX amino acid sequence predicts a mass of 99 kDa after cleavage of the signal peptide and protease cleavage. The mass of purified ATX assayed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry is 104,133 Da, consistent with a mature, glycosylated form of the enzyme (41).

Figure 1. Steady-state ATX-dependent hydrolysis of pNP-TMP and FS-3. A, time courses of pNP-TMP hydrolysis by 75 nmol/L ATX assayed by absorbance at 405 nm. The curves represent (bottom to top) 0, 0.1, 0.6, 0.9, 1.5, 3, 7, 15, 20, 30, and 40 nmol/L pNP-TMP. B, [pNP-TMP] dependence of hydrolysis rate. Solid line, best fit to a rectangular hyperbola (Eq. A). The $K_M$ for pNP-TMP is 1.4 mmol/L and the $k_{cat}$ for hydrolysis is 1.6 s⁻¹. C, time courses of FS-3 hydrolysis by 200 nmol/L ATX assayed by fluorescence ($\lambda_{ex} = 485 nm; \lambda_{em} = 520 nm$). The curves represent (bottom to top) 0, 1, 2, 4, 24, and 36 μmol/L of FS-3. D, [FS-3] dependence of hydrolysis rate. Solid line, best fit to a rectangular hyperbola. The $K_M$ for FS-3 is 4.5 μmol/L and the $k_{cat}$ was normalized to compare multiple days. Points, mean ($n = 4$); bars, SD.
ATX Expression in Normal Skin, Benign Nevi, and Malignant Melanoma

The overexpression of ATX in a variety of human malignancies is well established by multiple complementary techniques (see Introduction). We sought immunohistochemical confirmation of ATX overexpression in human melanoma using polyclonal antibodies raised against recombinant ATX protein. Human tissue microarrays containing 10 examples of normal skin, benign nevi, primary melanoma, or metastatic melanoma were stained with polyclonal ATX antibodies. Normal skin and benign nevi exhibit no increased expression of ATX, whereas 20% (2 in 10) of the primary and metastatic melanoma sections display strong cytoplasmic reactivity for the protein (Supplementary Fig. S1).7

Steady-State Enzymatic Activity of ATX

The nucleotide PDE activity of ATX was measured using pNP-TMP (36), a modified nucleotide with a phosphodiester bond that is hydrolyzed by ATX to yield p-nitrophenylate (Fig. 2A). Time courses of ATX-dependent pNP-TMP hydrolysis are linear over the time scale measured (30 min; Fig. 1A). The hydrolysis rate (v) depends hyperbolically on the pNP-TMP concentration (Fig. 1B). The best fit of the data to Eq. A yields a $K_M$ value of 1.4 ($\pm$0.1) mmol/L and a $k_{cat}$ value of 1.6 ($\pm$0.1) s$^{-1}$.

The lysoPLD activity of ATX was measured using the fluorescent lysophosphatidylcholine analogue FS-3 as the substrate (Fig. 2B; refs. 36, 37). Time courses of FS-3 hydrolysis are linear over the time scale examined (15 min; Fig. 1C). The hydrolysis rates follow Michaelis-Menton kinetics and depend hyperbolically on the FS-3 concentration (Fig. 1D). The best fit of the data to Eq. A yields a $K_M$ value of 4.5 ($\pm$0.6) $\mu$mol/L.

7 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Assay conditions were examined to ensure that inhibitory effects were not due to loss of the required metal ions of ATX. Changes in ATX activity as a result of both Ca\(^{2+}\) and Mg\(^{2+}\) were measured with pNP-TMP and FS-3 substrates. The apparent metal binding affinities with both substrates are <5 nmol/L (data not shown). Based on our binding affinities for Mg\(^{2+}\) and Ca\(^{2+}\) as well as published metal affinities for hexachlorophene and bithionol, inhibitors that chelate metal ions (discussed below; ref. 42), enough metal ions remained in the assay buffer for full ATX activity. Thus, metal chelation is not the cause of the observed inhibition.

### Table 1. Summary of ATX steady-state variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>No inhibitor</th>
<th>Hexachlorophene</th>
<th>Merbromin</th>
<th>Bithionol</th>
<th>NSC 48300</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_s) (pNP-TMP, mmol/L)</td>
<td>1.4 ± 0.1*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(k_{cat}) (pNP-TMP, s(^{-1}))</td>
<td>1.6 ± 0.1†</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(K_S) (FS-3, mmol/L)</td>
<td>4.5 ± 0.64†</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(K_i) (pNP-TMP, mmol/L)</td>
<td>—</td>
<td>15 ± 1.8(^i)</td>
<td>43 ± 7.5(^i)</td>
<td>60 ± 29(^i)</td>
<td>0.026 ± 0.0017**</td>
</tr>
<tr>
<td>(k_i) (pNP-TMP, mmol/L)</td>
<td>—</td>
<td>—</td>
<td>43 ± 7.5(^i)</td>
<td>180 ± 66(^i)</td>
<td>—</td>
</tr>
<tr>
<td>Inhibition type</td>
<td>—</td>
<td>Competitive</td>
<td>Noncompetitive</td>
<td>Mixed</td>
<td>Competitive</td>
</tr>
<tr>
<td>(K_i) (FS-3, mmol/L)</td>
<td>—</td>
<td>68 ± 9.2(^i)</td>
<td>—</td>
<td>66 ± 5.9</td>
<td>0.240 ± 0.045††</td>
</tr>
<tr>
<td>(k_i) (FS-3, mmol/L)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Inhibition type</td>
<td>—</td>
<td>Competitive</td>
<td>—</td>
<td>Competitive</td>
<td>Competitive</td>
</tr>
</tbody>
</table>

**NOTE:** Conditions: 50 mmol/L Tris, 5 mmol/L KCl, 140 mmol/L NaCl, 1 mmol/L MgCl\(_2\), and 1 mmol/L CaCl\(_2\) at pH 8.0, 25°C.

\(^*r^2 = 0.94\), degree of freedom (df) = 17.
\(^†r^2 = 0.93\), df = 37.
\(^ih^2 = 0.96\), df = 29.
\(^ir^2 = 0.93\), df = 101.
\(^kr^2 = 0.83\), df = 86.
\(^sr^2 = 0.81\), df = 45.
\(^**r^2 = 0.93\), df = 19.
\(^c^2 = 0.88\), df = 29.
\(^k^2 = 0.87\), df = 14.

### Inhibitor Screen

We hypothesized that the enzyme would be amenable to small-molecule inhibition given the nature of the products and reactants of ATX, and therefore, small-molecule libraries were screened in a high-throughput manner using robotics available at the Yale Chemical Genomics Facility. Initial screens used either pNP-TMP or FS-3 (monitoring the PDE and lysoPLD activity of the enzyme, respectively) as a single active site is reportedly responsible for both activities (2). The GenPlus library high-throughput screen yielded the three hits: hexachlorophene, bithionol, and merbromin (Fig. 2C).

![Figure 3](https://example.com/fig3.png)

**Figure 3.** Inhibitor effects on ATX lysoPLD activity. **A,** time courses of FS-3 hydrolysis in the presence of NSC 48300 at 2 μmol/L FS-3. **Top to bottom,** 0, 0.5, and 1 μmol/L of NSC 48300. **B,** [hexachlorophene] dependence of ATX activity. **Top to bottom,** 0, 200, and 300 μmol/L of hexachlorophene. **Solid lines,** best fits of averaged data points to rectangular hyperbolas. **Points,** mean (n = 4); **bars,** SD. The data have been corrected for internal filtering. **C,** [bithionol] dependence of ATX activity. **Top to bottom,** 0, 150, and 200 μmol/L. **Solid lines,** best fits of averaged data points (n = 2) to rectangular hyperbolas. **Points,** mean; **bars,** SE. **D,** [NSC 48300] dependence of ATX activity. **Top to bottom,** 0, 0.5, and 1 μmol/L. **Solid lines,** best fits of averaged data points (n = 2) to rectangular hyperbolas. **Points,** mean; **bars,** SD.
The National Cancer Institute Structural Diversity set yielded 10 hits. We confirmed inhibition by the three GenPlus hits and NSC 48300 from the National Cancer Institute collection with purified ATX and evaluated effects on cells.

**Inhibitory Effects on PDE Activity of ATX**

Hexachlorophene (Fig. 2C) slows autotaxin activity in a concentration-dependent manner (Supplementary Fig. S2A).\(^{7}\) The apparent pNP-TMP \(K_M\) value increases with hexachlorophene concentration, whereas the \(k_{cat}\) value is not significantly affected (Supplementary Fig. S2A).\(^{7}\) The best fit of the data to the general steady-state equation (Eq. B) yields \(x > 45\), consistent with competitive inhibition of pNP-TMP binding. The hexachlorophene affinity (\(K_I\)) obtained from the best fit of the data to the competitive inhibition equation (Eq. C) is 15 (±2) \(\mu\)mol/L (Table 1).

Merbromin (Fig. 2C) also inhibits ATX activity in a concentration-dependent manner. The apparent \(k_{cat}\) decreases with increasing merbromin, whereas the apparent \(K_S\) is unaffected, indicative of noncompetitive inhibition (Supplementary Fig. S2B).\(^{7}\) The best fit of the data to the noncompetitive inhibition equation (Eq. D) yields a merbromin \(K_I\) value of 43 (±8) \(\mu\)mol/L (Table 1).

Bithionol (Fig. 2C) inhibits pNP-TMP hydrolysis in a [bithionol]-dependent manner. The apparent pNP-TMP \(K_M\) value increases with bithionol concentration and the apparent \(k_{cat}\) decreases with bithionol, consistent with a mixed, noncompetitive inhibition mechanism (Supplementary Fig. S2C).\(^{7}\) The \(K_I\) for bithionol binding to ATX calculated from the best fit of the data to the mixed steady-state inhibition equation (Eq. B) is 60 (±29) \(\mu\)mol/L and the \(x\) value is 3.0 (±2.3; Table 1).

NSC 48300 (Fig. 2D) inhibits ATX activity in a concentration-dependent manner. The apparent \(K_M\) increases in a [NSC 48300]-dependent manner, whereas the apparent \(k_{cat}\) is unaffected, indicative of competitive inhibition (Supplementary Fig. S2D).\(^{7}\) The \(K_I\) for NSC 48300 binding ATX calculated from the best fit of the data to the competitive inhibition equation (Eq. C) is 47.5 nmol/L (Table 1).

Analogues of hexachlorophene, merbromin, and bithionol were identified based on structural motifs. The hexachlorophene analogue 2,2'-methylenebis(4-chlorophenol) (Fig. 2C) inhibits ATX noncompetitively with a \(K_I\) value of 104 (±15) \(\mu\)mol/L. Eosin Y (Fig. 2C), the merbromin analogue, inhibits ATX competitively with a \(K_I\) value of 47.5 nmol/L.

**Figure 4.** Effect of inhibitors on melanoma cell migration. A, cell migration of melanoma cells 4 h after the addition of (from left to right) 28 \(\mu\)L medium, 75 nmol/L LPA, or 50 nmol/L ATX. B, average of nine melanoma cell motility assays with medium, medium and 0.1% DMSO, 75 nmol/L LPA, or 50 nmol/L ATX ± inhibitors in the presence and absence of LPA. Columns, means; bars, SD. The addition of LPA to the experimental groups containing inhibitors (LPA rescue) completely abrogated the small-molecule inhibitory effect on cell motility (see Discussion). The LPA rescued motility was significantly increased over the inhibited motility for all inhibitors (\(P \leq 0.001\)). The increase in motility with both LPA and ATX was statistically significant (\(P \leq 0.0001\)). C, average melanoma cell motility as a function of inhibitor concentration. Left, inhibitors merbromin (black), bithionol (purple), hexachlorophene (red), and NSC 48300 (blue). Solid line, best fit to a rectangular hyperbola, which was only modeled for NSC 48300, as the other inhibitors could not be fit to a single binding event (see Discussion). Right, average melanoma cell motility as a function of blebbistatin concentration in the presence of 50 nmol/L ATX (black) or 75 nmol/L LPA (grey). Solid line, best fit to a rectangular hyperbola, modeled to a single binding event.
value of 9.6 (±1.8) μmol/L. RJC 03297 (Fig. 2C), the bithionol analogue, does not inhibit ATX at concentrations up to 100 μmol/L.

Inhibition of ATX lysoPLD Activity

The ability of these compounds to inhibit the lysoPLD activity of ATX was evaluated using FS-3. Hexachlorophene is a competitive inhibitor of ATX lysoPLD activity (Fig. 3B), with a $K_i$ value of 68 (±9) μmol/L (Table 1). Bithionol competitively inhibits ATX lysoPLD activity (Fig. 3C), with a $K_i$ value of 66 (±6) μmol/L (Table 1). The similarity between the $K_i$ values with the pNP-TMP and FS-3 substrates (60 and 66 μmol/L, respectively) is consistent with traditional competitive inhibition because the $K_i$ reflects binding to free ATX and should be independent of the substrate. NSC 48300 (Fig. 2D) competitively inhibits ATX lysoPLD activity (Fig. 3A and D), with a $K_i$ value of 240 (±45) nmol/L (Table 1). Among the GenPlus screen inhibitor analogues [eosin Y, 2,2'-methylenebis(4-chlorophenol), and RJC 03297], only eosin Y (an analogue of merbromin) inhibits ATX lysoPLD activity. Inhibition is noncompetitive, with a $K_i$ value of 116 (±37) μmol/L. Merbromin interferes with the fluorescent signal of the assay (data not shown), so its effect on lysoPLD activity could not be determined.

Inhibition of ATX lysoPLD activity by several NSC 48300 analogues was qualitatively compared in a time course assay by measuring the production of fluorescent product in the presence or absence of 10 μmol/L inhibitor over a 30-min interval. The parent compound was found to have nearly complete inhibition with 98.0% less product formation when compared with the enzyme alone. Deletion of an arylarsonic acid and the connected benzene ring weaken inhibition (NSC 10881; Fig. 2D). Substituting the arylarsonic acids with carboxic acids (NSC 86629; Fig. 2D) or removal of the linker so the benzene rings are directly bound (NSC 13792; Fig. 2D) eliminates inhibition. In the absence of the linker, replacing the arylarsonic acids with carboxic acid again eliminates inhibition (NSC60016; Fig. 2D), but inhibition can be partially recovered by moving the carboxic acid groups to the meta-position (NSC78785; Fig. 2D).

ATX-Induced Melanoma Cell Motility and Invasion and Its Inhibition

To verify that the ATX inhibitors were capable of affecting cell motility and invasion, we did Boyden chamber cell migration assays and Matrigel cell invasion assays with melanoma, breast, and ovarian cancer cells. ATX (50 nmol/L) or LPA (75 nmol/L) greatly stimulates A2058 melanoma cell migration in Boyden chamber assays and Matrigel invasion assays (Figs. 4 and 5). The number of migrating cells per high-power field increases >20-fold over medium alone, and the number of invading cells per high-power field increases by 3.5-fold. DMSO (final concentration of 0.3%) does not affect melanoma cell motility or invasion (Fig. 4A and B).

The ATX-induced stimulation of motility and invasion is inhibited in a dose-dependent manner by the ATX inhibitors hexachlorophene, bithionol, merbromin, and NSC 48300. Normal levels of motility and invasion in the

![Figure 5](http://example.com/figure5.png)
Small-Molecule Inhibitors of Autotaxin

Mol Cancer Ther 2008;7(10) . October 2008

Mol Cancer Ther 2008;7(10). October 2008

presence of inhibitors are regained with the addition of 75 nmol/L LPA (Figs. 4B and 5), verifying that the cells are still motile but that their reduced motility is secondary to an absence of LPA. The reduction in motility and invasion by some of the inhibitors could not be fit to a single binding event, as expected from the solution kinetics. This observation is explored in more detail in the discussion below.

Blebbistatin inhibition of ATX-induced cell motility could be experimentally modeled to a single high-affinity site resulting in an EC$_{50}$ of 54 μmol/L, consistent with EC$_{50}$ values of ~50 μmol/L reported for blebbistatin inhibition of adenocarcinoma cell motility (Fig. 4C; ref. 43). The inhibitory effects of blebbistatin could not be recovered by the addition of LPA (EC$_{50}$ = 27 μmol/L; Fig. 4C), suggesting that blebbistatin inhibits cell motility downstream of LPA.

To determine if the stimulation of melanoma migration was specific to a single melanoma cell line, we did cell migration assays on a panel of melanoma cell lines in the presence and absence of the enzymatic product of ATX, LPA. Two of the five cell lines were responsive to LPA presence and absence of the enzymatic product of ATX, was specific to a single melanoma cell line, we did cell streaming of LPA.

In the presence of inhibitors could be rescued with the addition of LPA (EC$_{50}$ = 27 μmol/L; Fig. 4C), supporting the notion that the inhibitory phenotype was linked to the ATX/LPA axis.

Discussion

The morbidity and mortality associated with melanoma is linked to its predisposition to metastasize. The prometastatic enzyme ATX was initially identified in melanoma cell culture and is associated with tumor aggression, invasion, and metastasis. ATX produces extracellular LPA, which binds to G protein–coupled receptors at the cell membrane and stimulates cell motility through the phosphoinositide 3-kinase pathway (44), a major signaling cascade deregulated in melanoma. Because of its ubiquity and extracellular location, it is an attractive molecular target in the prevention of metastatic melanoma. To determine whether the enzyme would be a tractable molecular target in the suppression of melanoma invasion and metastasis, we expressed and purified recombinant human protein and used this reagent to develop polyclonal antibodies and to identify small-molecule inhibitors. Our results indicate that expression of ATX is increased in primary and metastatic melanoma relative to benign skin and nevi and that small-molecule ATX inhibitors suppress melanoma migration and invasion.

Tissue microarrays of normal skin, nevi, and primary and metastatic melanoma stained with chicken polyclonal ATX antibodies show strong tissue overexpression in a portion (20%) of the primary and metastatic melanoma sections compared with benign nevi and normal skin (Fig. 1). Although clinical data correlating ATX expression with melanoma progression are not yet available, ATX expression has been linked to aggressive breast cancer (20), and future immunohistochemical studies with melanoma tissue linked to clinical data may shed light on the correlation between ATX and metastatic melanoma.

Two limited high-throughput screens identified viable small-molecule leads with affinities from low micromolar to low nanomolar (Fig. 2; Table 1); analogues of three of these molecules were also evaluated. These seven small-molecule inhibitors modulate the PDE enzymatic activity of ATX by competing with substrate binding to free ATX (i.e., they are competitive inhibitors). Six of these inhibitors, with the exception of 2,2′-methylenebis(4-chlorophenol), also display this behavior with the lysoPLD activity. Bithionol, merbromin, and 2,2′-methylenebis(4-chlorophenol) additionally display inhibitory activity by binding the ATX-pNP-TMP complex (i.e., they display uncompetitive, noncompetitive, and/or mixed inhibition). Some inhibitors [bithionol, eosin Y, and 2,2′-methylenebis(4-chlorophenol)] bind either the ATX-FS-3 complex or the ATX-pNP-TMP complex but not both. Hexachlorophene and NSC 48300 competitively inhibit PDE and lysoPLD activities with different K$_I$ values, unexpected in true competitive inhibitors that bind free enzyme, suggesting formation of a temporary ATX-substrate-inhibitor complex. Evaluation of multiple NSC 48300 analogues suggests that large, electronegative groups below the plane of the greasy center of the molecule are essential for inhibition.

All four inhibitors reduce melanoma cell migration in a dose-dependent fashion when assayed using the in vitro cell migration and invasion systems. Base levels of melanoma motility are recovered by the addition of LPA, supporting the notion that the inhibitors decrease tumor motility and invasion by decreasing the lysoPLD activity of ATX and not through nonspecific toxic effects on the cells or secondary effects on proteins outside the ATX/LPA pathway. Furthermore, the demonstration that the ATX inhibitors reduce cell invasion in breast and ovarian cancer cells suggests a role for ATX in other malignancies where ATX expression is reported to be increased, and shows that these effects are not an artifact of a particular cell line or tumor type.

Inhibition of cell motility and invasion is biphasic (Figs. 4C and 5), with some (~40%) reduction occurring at inhibitor concentrations lower than those needed to modulate ATX activity in solution (Figs. 3 and 4); complete inhibition occurs over a range comparable with that needed to inhibit ATX (i.e., with apparent K$_I$ in micromolar range).
We interpret this behavior to reflect the differential partitioning of ATX among the lipid and aqueous phases, which yield deviations from solution steady-state behavior (45). We can eliminate the possibility that this behavior is nonspecific and arises from an LPA/ATX-independent pathway because the inhibitors do not affect the cell motility of tumor cells unresponsive to ATX and LPA stimulation (e.g., mouse melanoma cell line B16). The inhibition by blebbistatin displays the expected kinetics for a soluble enzyme/substrate system, proving that the kinetic behavior discussed above is not an artifact of the assay, and also shows that myosin II is a downstream component of the LPA signaling pathway that leads to cell motility.

These experiments validate ATX as a molecular target for metastatic melanoma and show the feasibility of developing high-affinity small-molecule inhibitors of ATX capable of inhibiting its melanoma migration and metastasis. These studies also point the way to in vivo studies, as one of the lead compounds (bithionol) has received Food and Drug Administration approval as a second-line orally administered medication for the treatment of helminthic infection that can be safely dosed in humans at 25 mg kg⁻¹ body weight per day (46). Detailed toxicology and pharmacokinetics on the drug are available, and oral doses of 50 mg/kg in three divided alternate daily doses for 5 days maintain serum levels in the range of 140 to 550 μM/L in rabbits, dogs, and humans, or several times greater than those required to observe a 50% inhibition of melanoma cell migration in our in vitro assays. In the case of NSC 48300, although the exposure to arylarsenic acids may arouse concern, pharmaceutically more acceptable alternatives may be found using elements within the same group of the periodic table (e.g., antimony), which are equally potent. These studies lay the groundwork for future in vivo experiments to test the extent to which ATX inhibitors will be useful in preventing melanoma spread and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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