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Anticancer activity of FTY720: phosphorylated FTY720 inhibits autotaxin, a metastasis-enhancing and angiogenic lysophospholipase D

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Abstract

FTY720 is an immunomodulator that is phosphorylated *in vivo* and inhibits lymphocyte mobilization by targeting sphingosine 1-phospate receptors. At doses higher than required for immunomodulation, FTY720 inhibits tumor progression through an unknown mechanism. Here we show that FTY720-phosphate is a competitive inhibitor (K_i ~0.2 μM) of autotaxin (ATX or NPP2), a nucleotide phosphodiesterase/pyrophosphatase (NPP) that enhances metastasis and angiogenesis and acts as a lysophospholipase D to produce the lipid mediator lysophosphatidic acid (LPA). FTY720-phosphate did no affect the activity of NPP1, the closest relative of ATX. After oral administration in mice, FTY720 (3 mg/kg) significantly reduced plasma LPA levels. These results suggest that FTY720 may exert its anticancer effects, at least in part, by targeting the ATX-LPA axis.

Keywords

FTY720; autotaxin; lysophosphatidic acid; tumor progression

1. Introduction

FTY720 (also known as fingolimod) is a novel immunomodulator that interferes with lymphocyte trafficking. This investigational drug is currently in Phase III clinical trials for patients with relapsing multiple sclerosis [1]. FTY720 is structurally similar to the natural lipid sphingosine and is phosphorylated *in vivo* to the active principle FTY720-phospate (FTY-P). FTY-P binds with high affinity to G protein-coupled receptors for the lipid mediator sphingosine 1-phosphate (S1P), particularly receptor subtype $S1P_1$ [2;3]. Upon FTY-P binding, $S1P_1$ receptors are targeted for internalization and degradation [4]. The “functional

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antagonist" hypothesis holds that lymphocytes exposed to FTY-P fail to respond to S1P and thereby remain sequestered in secondary lymphoid organs thus being unable to migrate to sites of inflammation [5].

In addition to its immunomodulating action, FTY720 exerts striking anticancer effects in animal models. When administered at doses at least 10-fold higher than required for immunomodulation, FTY720 (3–10 mg/kg/d) inhibits tumor growth, angiogenesis and metastasis in various carcinoma models [6–9] and the syngeneic B16 melanoma model [10]. Furthermore, FTY720 remarkably suppresses Bcr/Abl-driven leukemogenesis in mice without overt toxicity [11] and it prolongs survival in a mouse model of disseminated B-cell malignancy [12]. However, the mechanism whereby FTY720 restrains tumor progression is unclear although various scenarios have been proposed, ranging from S1P receptor modulation to selective induction of apoptosis [6–12].

We previously reported a novel action of S1P, namely as an inhibitor of autotaxin (K_i ~0.1 μM) [13]. Autotaxin (ATX, also known as NPP2) is a secreted nucleotide phosphodiesterase/pyrophosphatase (NPP) [14] that primarily functions as a lysophospholipase D (lysoPLD), producing the lipid mediator lysophosphatidic acid (LPA) from more complex lysophospholipids [15;16]. LPA acts on specific G protein-coupled receptors to stimulate the proliferation, migration and survival of many cell types, both normal and malignant [17–19], and is an effector of tumor progression in mice [20;21]. Like its product LPA, ATX is strongly implicated in tumor progression: it is overexpressed in various cancers (reviewed in [16;18]), it promotes tumor aggressiveness, metastasis and angiogenesis in preclinical models [22;23], while a pharmacological inhibitor of ATX suppresses melanoma metastasis [24]. Moreover, gene targeting studies in mice have uncovered a vital role for ATX in vascular development [25;26]. Thus, the ATX-LPA axis is an attractive target for anticancer and anti-angiogenesis therapy.

Given the structural similarity of FTY-P to S1P, we sought to determine whether FTY-P can mimic S1P in inhibiting ATX. Here we report that FTY-P is a competitive inhibitor of ATX *in vitro* and that the parent drug reduces circulating LPA levels *in vivo*. We suggest that the anticancer effects of FTY720 may be attributed, at least in part, to inhibition of the ATX-LPA axis by FTY-P.

2. Materials and Methods

2.1. Recombinant ATX

Human ATX cDNA was cloned with a C-terminal His tag in pcDNA3 (InVitrogen). HEK293T cells were transfected with pcDNA3-ATX-His and maintained in serum-free DMEM for 48 hrs. Recombinant ATX was purified from conditioned medium using TALON Resin beads (Clontech).

2.2. ATX activity assays

The phosphodiesterase activity of ATX toward *para*-nitrophenyl tymidine-*5'*-monophosphate (pNP-TMP) was measured by light absorbance in 96-well plates, as described previously [13]. ATX-His (75 nM) was added to 100 μl of Tris-buffered saline (pH 8.0) containing 1 mg/ml fatty-acid free BSA and 1 mM pNP-TMP. After incubation for 30 min. at 37 °C, the amount of liberated *para*-nitrophenol was quantified by measuring the absorbance at 405 nm. To measure lysoPLD activity, the fluorigenic lysophospholipid substrate FS3 (2.5 μM) was used in Tris-buffered saline (pH 8.0) containing 1 mg/ml fatty-acid free BSA. After addition of ATX-His (75 nM), the increase in fluorescence as monitored at 37 °C (excitation, 485 nm; emission, 520 nm) as described previously [27].

2.3. Studies in mice

Female mice (C57Bl/6 × sv129) were dosed daily for five days by oral gavage, 0.2 ml either with vehicle of 3.0 mg/kg FTY720. At day 6, mice were sacrificed 24 hrs after the final dose. Citrate-treated plasma was extracted by acidic butanol and LPA quantified using a highly sensitive radio-enzymatic assay based on the transfer of a [^{14}C] fatty acyl chain onto LPA resulting in the production of [^{14}C] phosphatidic acid [28]. Lymphocytes were counted as described [3].

3. Results and discussion

3.1. Inhibition of ATX activity

ATX/NPP2 uses a single catalytic site for the hydrolysis of both lipid and non-lipid substrates [14]. We measured the nucleotide phosphodiesterase and lysoPLD activity of recombinant ATX using pNP-TMP and FS3 as substrates, respectively. We previously found that ATX is inhibited by LPA and S1P (Ki ~ 0.1 uM) [13]. We examined the effect of FTY-P (the bioactive *S*-enantiomer; Fig. 1A) and compared its effect to that of S1P. FTY-P inhibited the catalytic activity of ATX in a dose-dependent manner using either a nucleotide or a lysophosphospholipid as substrate (Fig. 1B,C). Using substrate concentrations close to the K_m values [13;27], FTY-P inhibited ATX activity with IC_{50} values of 0.3–0.4 μM. FTY-P was found to be somewhat less potent than S1P (IC_{50} ~ 0.1 μM) (Fig.1B,C; see also ref. [13]). Substrate titration curves and double-reciprocal plot analysis revealed that FTY-P is a competitive inhibitor, producing an increase in K_m without affecting V_{max} (Fig. 1D). The inhibition constant (K_i) was approx. 0.2 μM, a value about two-fold higher than that for S1P [13]. The parent compound (FTY720, 1 μM) had no discernible effect on ATX activity (Fig. 2).

3.2. Selectivity

ATX/NPP2 is the only NPP family member with lysoPLD activity [14]. The inhibitory effect of FTY-P was selective since the compound did not affect the activity of NPP1, the closest relative of ATX/NPP2, nor did it inhibit a broad-specificity phosphodiesterase (Fig. 2). This implies that ATX has a unique binding site for FTY-P, not present in NPP1. In this respect we note that, apart from its catalytic site (residue Thr210), ATX contains an additional substrate-binding sequence (aa 318–334) not found in other NPP family members [29]. It will be interesting to determine whether this region may serve as a binding site for FTY-P.

3.3. Administration in mice

ATX is the major LPA-producing enzyme *in vivo*, as shown by the finding that half-normal ATX expression results in 50% reduced plasma LPA levels [25;26]. Therefore, pharmacological inhibition of ATX *in vivo* is expected to lower plasma LPA levels. In a mouse cancer model, drug-induced depletion of plasma LPA is accompanied by strongly reduced bone metastasis, supporting the notion that circulating LPA is a determinant of tumor progression [20]. To examine whether FTY720 may affect plasma LPA levels, we treated mice orally once daily with 3 mg/kg FTY720. After 5 days, the number of circulating lymphocytes and plasma LPA levels were determined. FTY720 administration caused significant lymphopenia (Fig. 3A) and, more importantly, an approx. 50% reduction in plasma LPA levels (from 240 to 110 nM), consistent with plasma ATX activity being inhibited by FTY-P (Fig. 3B).

3.4. Concluding remarks

Inhibition of the ATX-LPA axis by FTY-P may provide a mechanistic explanation for the reported anticancer effects of FTY720. Although the doses at which FTY-P inhibits ATX activity are substantially higher than those required to target S1P receptors and arrest

lymphocyte migration, they are within the predicted pharmacological range obtained after high FTY720 dosage. The blood levels of FTY-P can exceed those of the parent compound several-fold [3]. Based on published data [3], we estimate that high FTY720 doses may result in FTY-P blood levels of about 1 μM, a concentration that inhibits ATX activity by approx. 70% (Fig. 1). Given the high doses needed, the anticancer activity of FTY720 is unlikely to be credited only to S1P receptor modulation but must involve alternative or additional targets such as the ATX-LPA axis identified here. Not only is the ATX-LPA signaling system strongly implicated in tumor progression [16–18;20–24], evidence for S1P receptors playing an active role in cancer is limited. Another possibility is that the antitumor activity of FTY720 may involve a (direct or indirect) action of the parent compound on intracellular targets such as, for example, the tumor suppressor protein phosphatase 2A (PP2A). FTY720 stimulates PP2A activity specifically in certain leukemic cells, albeit at high concentrations (2.5–10 μM), and thereby may enhance apoptosis and suppress leukemogenesis [11;12;30]. The present results lead us to suggest that the anticancer effects of FTY720 may relate to FTY-P inhibiting the ATX-LPA axis, possibly in conjunction with other tumor-suppressing actions, a scenario that clearly warrants further investigation.

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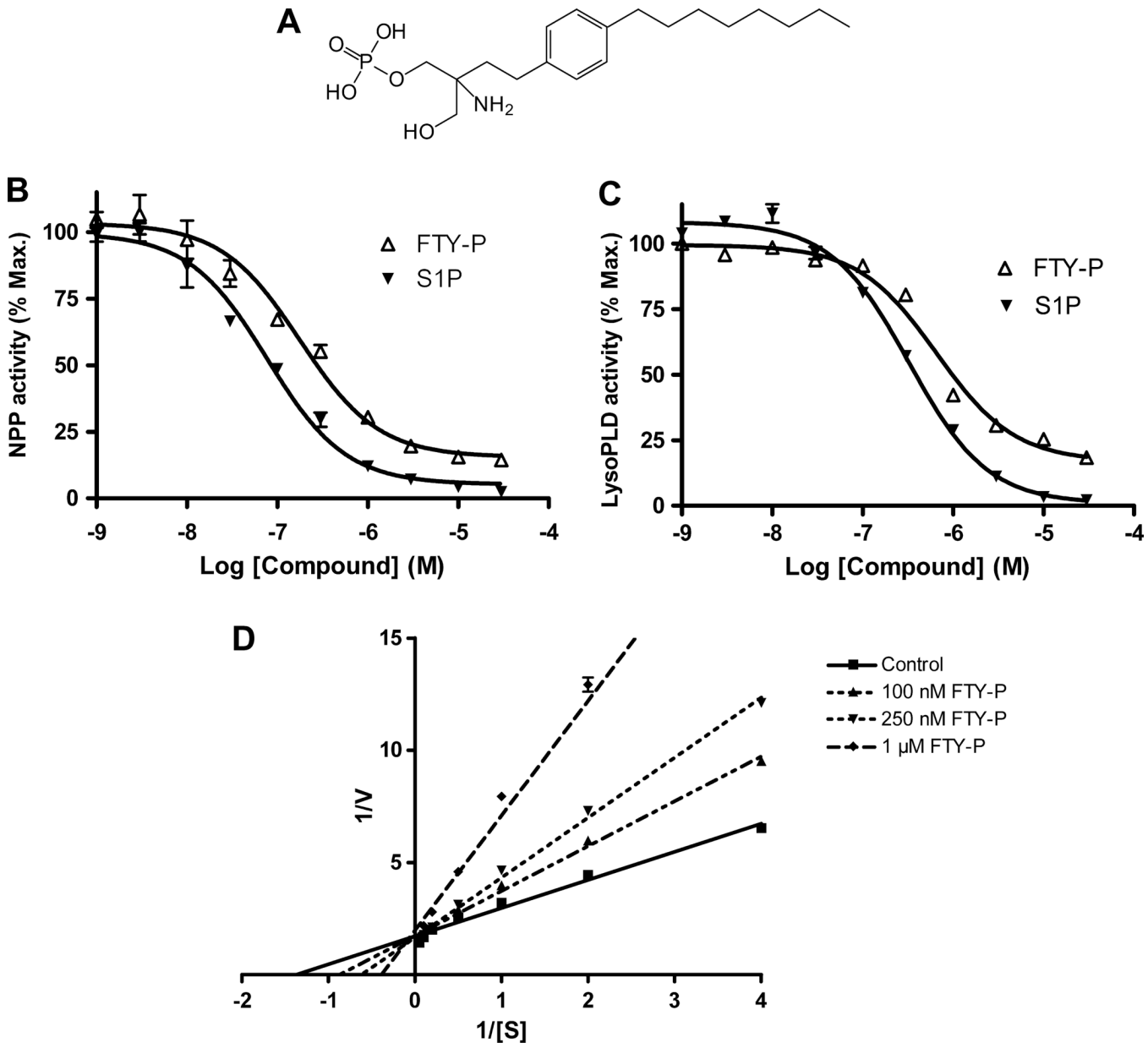

Fig. 1. Inhibitory effects of FTY-P on ATX activity

(A) Structure of FTY-P. (B) Nucleotide phosphodiesterase (NPP) activity of recombinant ATX (70 nM) using pNP-TMP (1 mM) as substrate. (C) LysoPLD acitivity of ATX using FS3 (2.5 μM) as substrate.

(D) Double-reciprocal plot analysis of ATX inhibition by FTY-P. FTY-P was added at the indicated concentrations and ATX catalytic rate (V) was determined using pNP-TMP (1 mM) as substrate (S). FTY-P-inhibited ATX shows the same Y-intercept as untreated ATX (Control), indicating that FTY-P is a competitive inhibitor.

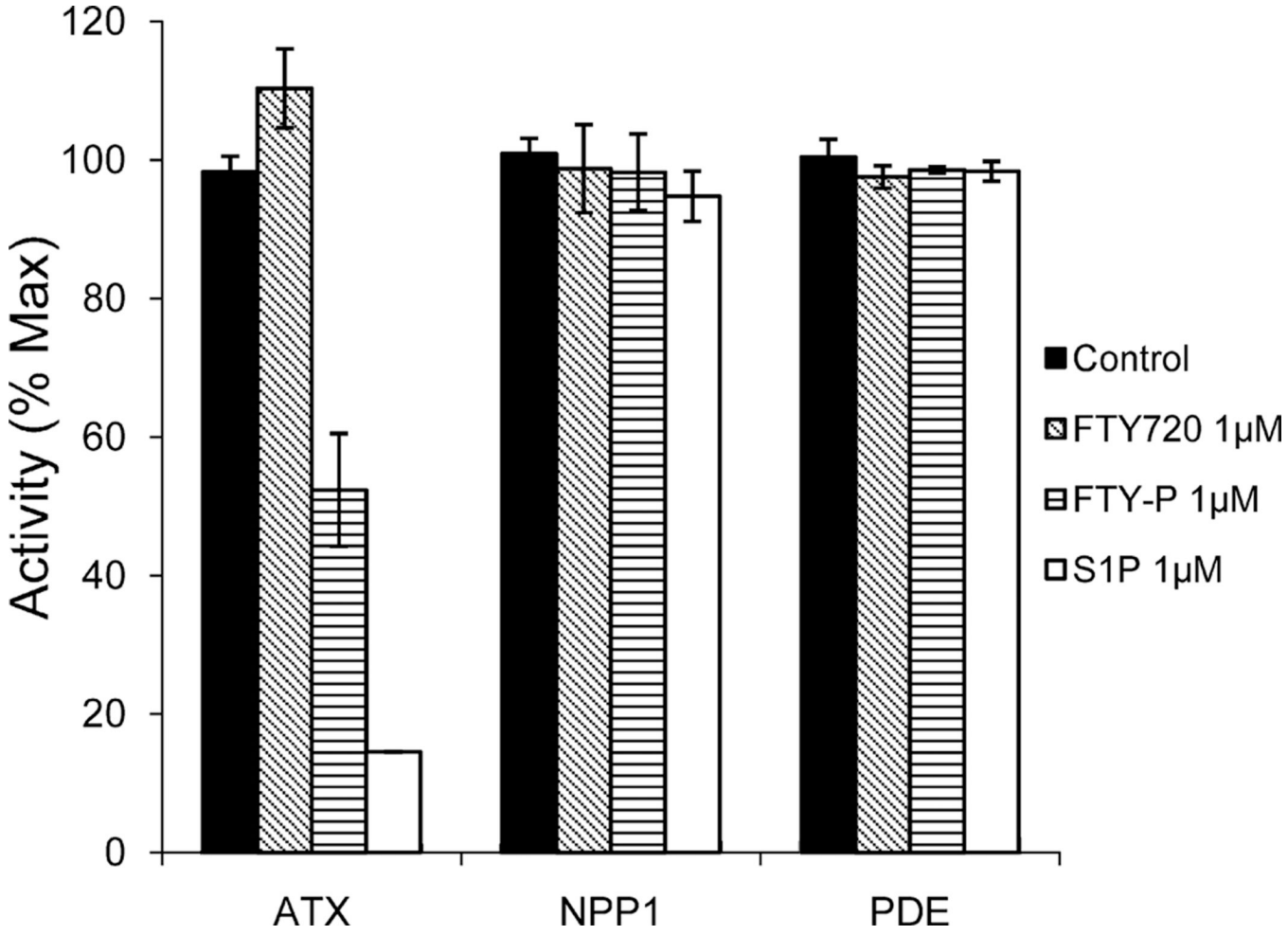

Fig. 2. Specificity of FTY-P and S1P toward ATX as measured by pNP-TMP hydrolysis

FTY720, FTY-P and S1P were used at the indicated concentrations. Purified NPP1 was used at 200 nM, as in a previous study [13]. PDE denotes a broad-specificity phosphodiesterase from snake venom (1 U/ml).

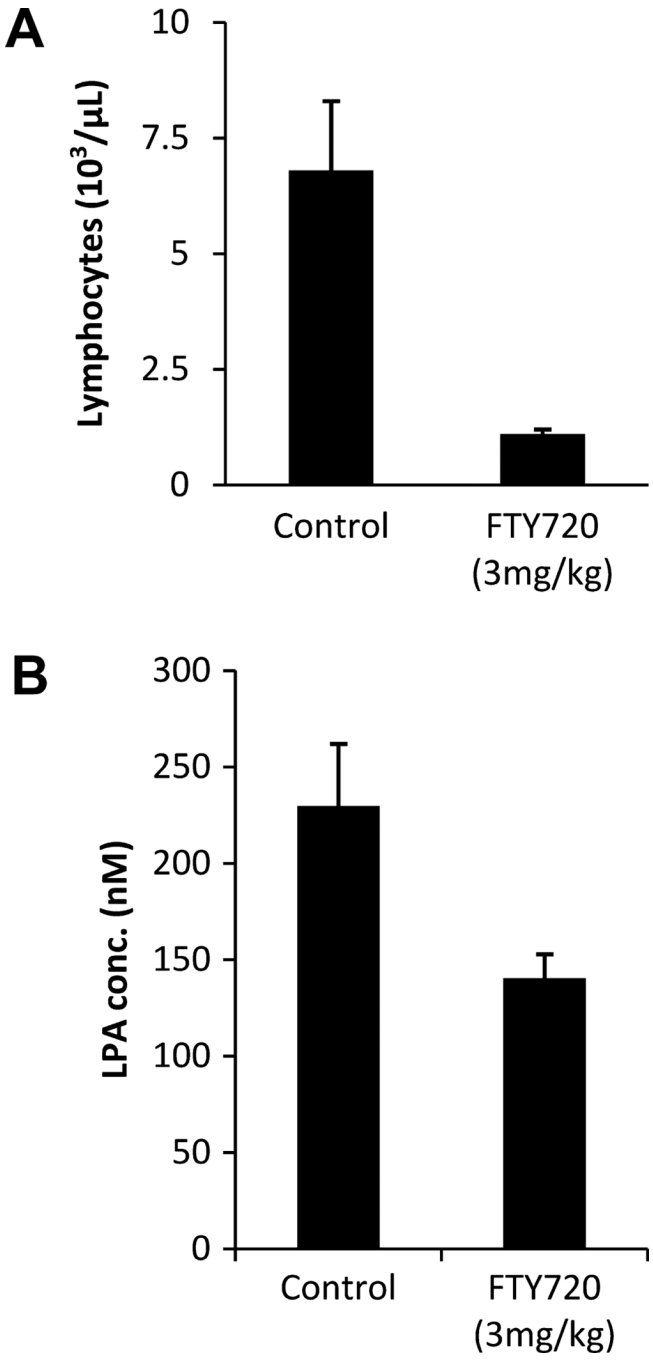

Fig. 3. Plasma LPA levels and blood lymphocyte counts in FTY720-treated mice

Three sets of 5 mice were dosed with vehicle and FTY720 (3.0 mg/kg) daily for 5–6 days. (A) Blood lymphocyte counts in FTY720- and vehicle-treated mice. (B) Plasma LPA levels (nM; $p<0.01$) in treated and non-treated animals, as determined by a radioenzymatic assay based on the transfer of a [^{14}C] fatty acyl chain onto LPA resulting in the production of [^{14}C] phosphatidic acid [28].