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Gilenya (FTY720) inhibits acid sphingomyelinase by a mechanism similar to tricyclic antidepressants

Glyn Dawson [a,b,*], Jingdong Qin [a]

[a] Department of Pediatrics, University of Chicago, Chicago, IL, USA
[b] Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, USA

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ABSTRACT

The immunomodulator drug Gilenya (FTY720), marketed as the first oral sphingosine-1-phosphate receptor (S1P-R) modulator for treatment of Multiple Sclerosis (MS) also inhibits lysosomal acid sphingomyelinase (ASMase). Treatment of cultured cells for 24 h with FTY720 (up to 10 μM) inhibited ASMase by >80% and this could be reversed by pre-treatment with the cathepsin protease inhibitor leupeptin (5 μM). In contrast, neutral sphingomyelinase activity was unaffected and sphingosine-1-phosphate treatment had no effect on ASMase. RT-PCR revealed no inhibition of ASMase mRNA and there was no direct (*in vitro*) inhibition of ASMase by either FTY720 or FTY720-phosphate. This suggests that its mechanism of inhibition is similar to that of tricyclic anti-depressants such as desipramine, which are also amphiphilic cationic drugs. Both Desipramine and FTY720 treatment reduced ASMase without significant inhibition of other lysosomal hydrolases but most hydrolases showed increased secretion (up to a 50% increase) providing more evidence of lysosomal disruption by these drugs.

1. Introduction

The $C_{19}H_{33}NO_2$ immunomodulator drug FTY720 (CH_3 $(CH_2)_7$-phenyl-$(CH_2)_2$-C (NH_2) (CH_2OH)–CH_2OH)) (Gilenya), is an analog of naturally occurring myriocin, which inhibits *de novo* sphingolipid synthesis [1]. In cultured non-neural cells it has been shown to inhibit ceramide synthase-2 [2,3], leading to a reduction in ceramide levels in one study [2]. It also appears to inhibit the *de novo* biosynthesis of sphingosine-1-phosphate (S1P) [2], a sphingolipid with roles in the regulation of cell growth, death, senescence, adhesion, migration, angiogenesis and intracellular trafficking [4–6]. FTY-720's therapeutic action in Multiple Sclerosis is to inhibit the exit of autoreactive memory T-cells from secondary lymphoid organs to produce a peripheral lymphopenia [7]. However, it also localizes to CNS white matter along the myelin sheath and gets converted to FTY720-P (presumably as the result of sphingosine kinase-2 action) after crossing the blood–brain barrier [2,8]. FTY720-P is believed to act as a functional S1P antagonist which induces complete internalization and desensitization of lymphocyte G-protein-coupled receptors, since it is not as easily degraded as S1P [7]. Because of its reported role in promoting remyelination [9], its clinical importance and observed side-effects it is important to understand the action of FTY720 in full. We now report that it is also a potent inhibitor of lysosomal acid sphingomyelinase (ASMase), most likely by a mechanism similar to desipramine and other tricyclic antidepressants [10–13].

Tricyclic antidepressants such as desipramine are believed to induce intracellular degradation of acid sphingomyelinase [10–12] and acid ceramidase [13] by interfering with the binding of the enzyme to the inner membranes of late endosomes and lysosomes. The effect is seen with cationic amphiphilic drugs at 5 μM but not by neutral or anionic ones [10–13]. Inhibition is typically not more than 90% so there is only modest accumulation of sphingomyelin and depletion of ceramide, unlike that observed in cells totally deficient in ASMase (Niemann–Pick disease) [14]. ASMase has an isoelectric point of 6.8 so is positively charged at lysosomal pH 4.5 and is believed to bind the lysosome/endosome-specific phospholipid BMP (LBPA) [11]. Displacement of ASMase from the membrane by cationic lipophilic drugs is hypothesized to expose ASMase to proteases [14] and the desipramine-induced degradation of ASMase (and ACeramidase) can be blocked by both leupeptin and CA074Me (a cathepsin B/L inhibitor) but not by pepstatin.

2. Materials and methods

2.1. Chemical and cell culture

2-Amino-2-[2-4(octylphenyl)ethyl] 1,3propanediol (FTY720) and 2-amino-2[2-(4-octylphenyl)ethyl]-1,3-propanediol, mono

* Corresponding author at: Departments of Pediatrics, Biochemistry and Molecular Biology, MC4068, University of Chicago, Chicago, IL 60637, USA. Fax: +1 773 702 9234.
E-mail address: dawg@uchicago.edu (G. Dawson).

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dihydrogen phosphate ester (FTY720-P) was obtained from Cayman Chem. Co. (Ann Arbor, MI); sphingosine-1-phosphate from Avanti Polar Lipids, Inc (Alabaster, Al) and leupeptin from Roche (Indianapolis, IN).

Neural-derived cells (LA-N-5 and HOG) and mouse skin fibroblasts were grown in monolayer culture in DMEM supplemented with 10% fetal calf serum as described previously [15]. Drugs were dissolved in ethanol and added to the culture medium to produce final concentrations of 5–15 μM.

2.2. Lysosomal hydrolase assays

Cells were harvested, the pellets resuspended and lysed in 25 mM Tris–HCl, 150 mM NaCl and 1% Triton X-100 pH 7.4 and aliquots (10–20 μg protein) and assayed with the appropriate 4MU or HMU substrate as described previously [16]. For ASMase the incubation was carried out at pH 4.5 in 150 mM sodium acetate buffer containing 1 mM EDTA to block any NSMase activity. The HMU released was followed fluorometrically in a 96-well FLX microplate reader. The enzyme activities were calculated from the slope of the graph of intrinsic fiscence plotted against time and standardized by μg of protein.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using a Qiagen total RNA extract kit (Valencia, CA), and RT-PCR was executed with an RT-PCR one step kit (Qiagen, Valencia, CA) using specific primer-pairs. Primers used were human ASMase, Forward 5′-CAGGGTTCCTGGCTGGGCAGCA-3′; Reverse 5′-GGTCCTGGACCATGAGACCTAC-3′; β-actin primer: Forward 5′-ATTGGCAATGAGCGGTTCC-3′; Reverse 5′-GGTAGTTTC GTGGATGCCACA-3′.

The reaction mixture was prepared in PCR tubes according to the kit menu and put into a Perkin Elmer GeneAMP PCR System 2400. The RT-PCR reaction was at 50 °C for 30 min, 95 °C for 15 min; then 94 °C, 60 °C, 72 °C, 1 min each, 35 cycles; 72 °C with 10 min for extension. The RT-PCR amplified samples were visualized on 1.2% Agarose gels using ethidium bromide.

2.4. Western blot analysis

Electrophoresed proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and Western blotting carried out with the anti-ASMase polyclonal antibody (Santa Cruz, Santa Cruz, CA) according to the manufacturer's instructions. Positive bands were detected with a chemiluminescence kit from Fisher Scientific (Pittsburgh, PA).

3. Results

3.1. FTY720 inhibited ASMase in a dose-dependent manner over the concentration range 0–10 μM

The inhibition of ASMase by FTY720 (Fig. 1A) was similar to that previously reported for desipramine (Fig. 1B); Representative other lysosomal hydrolases (β-D-hexosaminidase (Fig. 1A) and β-D-glucosidase (Fig. 1B) were not inhibited.

3.2. FTY720 and inhibition of ASMase is blocked by the co-addition of leupeptin

Treatment of LA-N-5 cells with FTY720 (10 μM) induced a 90% inhibition of ASMase activity, reversible with co-addition of leupeptin (5 μM) (FTY + Leu) (Fig. 2). Leupeptin alone (5 μM) stimulated ASMase but S1P (10 μM) had no effect. Similarly, Myriocin,

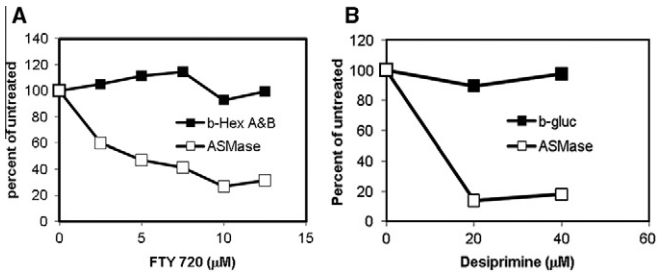

Fig. 1. Dose–response of inhibition of ASMase by FTY-720. Cells were incubated with FTY720 (Panel A) or desipramine (Panel B) at the concentration indicated for 24 h, harvested, lysed and the specific activity of ASMase and other lysosomal hydrolase activities (β-D-hexosaminidase) (A) and β-D-glucuronidase (B) were determined as described in the text.

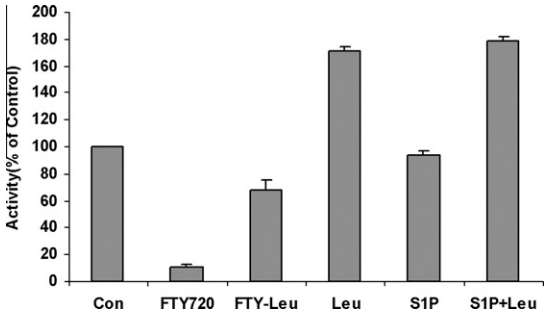

Fig. 2. FTY720 (but not S1P) inhibits ASMase in cultured cells, and leupeptin (Leu) can reverse the inhibition. FTY720 and S1P were added at 10 μM and leupeptin at 5 μM as described in the text. Results were based on multiple assays run in triplicate and are expressed as percent of control activity.

which is a zwitterionic, atypical amino acid (and therefore not an amphiphilic cation), did not inhibit ASMase activity under similar conditions.

Similar results were observed in other cell lines (e.g. HOG and mouse fibroblasts, data not shown).

3.3. FTY720 had no effect on mRNA levels for ASMase but reduced ASMase enzyme protein

Treatment of cells with FTY720 (10 μM) did not have any effect on mRNA levels (Fig. 3A) but reduced ASMase protein levels in parallel with the reduction in enzyme activity (Fig 3B).

3.4. Neither FTY720 nor FTY720 phosphate inhibited ASMase in vitro

Since phosphates are potent non-competitive ASMase inhibitors *in vitro* [16] we compared the effect of FTY720 and FTY720-P

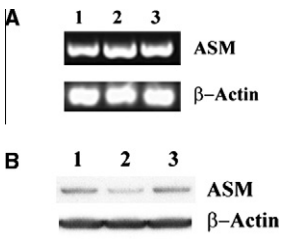

Fig. 3. FTY720 does not affect the message levels for ASMase even when inhibiting activity >90%. Panel A: RT-PCR of human LA-N-5 cells. Lane 1, control; lane 2, 10 μM FTY720 treatment; Lane 3, 5 μM leupeptin pre-incubation for 1 h followed by 10 μM FTY720 treatment. β-Actin is the control for sample application. Panel B: Western blot of human LA-N-5 cells. Lane1, control; Lane2, 10 μM FTY720 treatment; Lane 3, 5 μM leupeptin pre-incubation for 1 h then 10 μM FTY720 treatment for 24 h. β-Actin is the control for sample application.

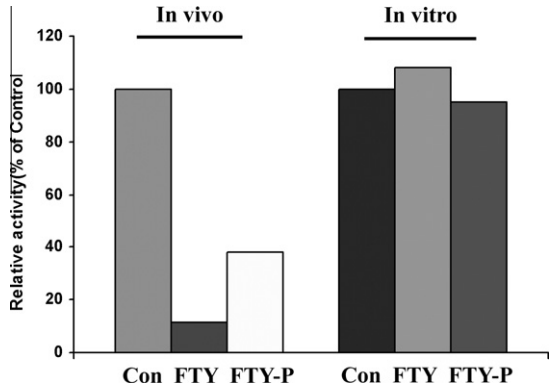

Fig. 4. FTY720 and FTY720 phosphate inhibit on ASMase *in vitro*. Both FTY720 and FTY720-P (10 μM) for 24 h inhibited ASMase *in vivo*. Treatment of homogenates with either drug at concentrations up to 50 μM and times up to 2 h did not result in any inhibition of ASMase activity, measured as described in the text.

(10 μM) in intact cells (24 h) with that in cell homogenates (up to 2 h) and saw no *in vitro* inhibition at concentrations up to 50 μM (Fig. 4). Treatment with chloroquine or ammonium chloride reduced the levels of most lysosomal hydrolases and increased their secretion (data not shown) but there was no specificity for ASMase.

4. Discussion

Our results suggested that FTY720 inhibits ASMase activity by a mechanism which involves the proteolytic degradation of the enzyme complex. In this regard it appears to be similar to the mechanism proposed for desipramine and amytryptaline action on ASMase and ACerase [11–14]. Although FTY720 acts predominantly on the immune system and is believed to exert other important actions on S1P receptors after being converted to FTY720-P, this conversion is very small (<1% [2]. FTY720 has been shown to affect the metabolism of a wide range of sphingolipids, possibly because it is structurally related to the inhibitor of *de novo* sphingolipid synthesis, myriocin [2,3]. However, neither myriocin nor S1P (which are zwitterionic) were inhibitory to ASMase, supporting the mechanism of amphiphilic cation action to release ASMase for degradation [11–14].

Unlike ceramide synthase-2 [2,3] we observed no direct *in vitro* inhibition of ASMase by FTY720 but 70–90% inhibition in whole cells. Analyses of FTY720-treated cells showed a modest 10% increase in sphingomyelin and confirmed that over the lower concentration range used (0–10 μM) there was very little decrease (10%) in ceramide levels [2]. The reported 2-fold decrease in both sphingosine and S1P [2] may result from the reported inhibition of ceramide synthase [2,3] and the likely inhibition of acid ceramidase which is sensitive to desipramine [12]. At high concentrations (250–500 μM), drugs such as Cocaine (a membrane-permeate weak base), are concentrated by acid tropic sequestration and rapidly inactivate acid sphingomyelinase, (a 10-fold decrease in V_{max} with identical K_m) as well as phospholipase A1 [17]. This was blocked by the proton ionophore, monensin, the vacuolar ATPase inhibitor, bafilomycin as well as cathepsin protease inhibitors such as leupeptin and E64a. High concentrations of cocaine also induced selective sphingomyelinase proteolysis and extensive lipid storage in lysosomes (electron-dense lamellar structures after 3 days) which was rapidly reversed by cocaine removal, suggesting fast ASMase turnover. Thus the effects of FTY720 could be cumulative.

5. Conclusion

Our novel finding is that FTY720 inhibits ASMase in a manner very similar to that of other amphiphilic cationic drugs known as functional inhibitors of acid sphingomyelinase [18]. Both FTY720 and FTY720-phosphate had no direct effect on ASMase but were able to expose the lysosomal ASMase to proteases, since ASMase degradation and loss of activity was blocked by pre-exposure to cathepsin inhibitors. Leupeptin alone also increased basal ASMase activity but had no effect on other lysosomal hydrolase activities, suggesting a uniquely high turnover rate for ASMase. A 10% increase in sphingomyelin and a 10% depletion of ceramide was observed which shows a functional effect of the inhibition might cumulatively increase when FTY720 is used over many years.

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