Antitumor activity of a novel S1P2 antagonist, AB1, in neuroblastoma

Mei-Hong Li, Rolf Swenson, Miriam Harel, Sampa Jana, Erik Stolarzewicz, Timothy Hla, Linda H. Shapiro and Fernando Ferrer

Center for Vascular Biology, University of Connecticut Health Center, Farmington, CT06030 (M-HL, LHR, FF); Arroyo Biosciences LLC, Princeton, NJ08540 (RS); Department of Urology and Surgery, Connecticut Children's Medical Center, Hartford, CT 06106 (MH, FF); TCG Life Sciences Limited, Hinjewadi, Pune 411057, India (SJ); Chem-Master International Inc., Stony Brook, NY11794 (ES); Center for Vascular Biology, Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, NY 10065 (TH)
JPET #224519

a) Running Title: Antitumor activity of a novel S1P2 antagonist AB1

b) Corresponding author: Fernando Ferrer, Department of Urology and Surgery, Connecticut Children’s Medical Center, 282 Washington Street, Hartford, CT, U Tel.: (860) 545-8847; Fax: (860) 545-9545; E-mail: fferrer@connecticutchildrens.org

c) Number of text pages: 26

Number of tables: 0

Number of figures: 9

Number of references: 33

Number of words in Abstract: 250

Number of words in Introduction: 495

Number of words in Discussion: 794

d) List of nonstandard abbreviations: CCL2, chemokine (C-C motif) ligand 2; CTGF, connective tissue growth factor; GB, glioblastoma; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NB, neuroblastoma; RIPA, radioimmunoprecipitation assay buffer; S1P, sphingosine-1-phosphate; TAM, tumor-associated macrophage

e) A recommended section: Drug Discovery and Translational Medicine
Abstract

The bioactive lipid sphingosine-1-phosphate (S1P) and its receptors (S1P<sub>1-5</sub>) play critical roles in many pathological processes including cancer. The S1P axis has become a bona fide therapeutic target in cancer. JTE-013, a known S1P<sub>2</sub> antagonist, suffers from instability <i>in vivo</i>. Structurally modified, more potent and stable S1P<sub>2</sub> inhibitors would be desirable pharmacological tools. One of the JTE-013 derivatives, AB1, exhibited improved S1P<sub>2</sub> antagonism compared to JTE-013. Intravenous pharmacokinetics indicated enhanced stability or slower clearance of AB1 <i>in vivo</i>. Migration assays in glioblastoma showed that AB1 was slightly more effective than JTE-013 in blocking S1P<sub>2</sub>-mediated inhibition of cell migration. Functional studies in the neuroblastoma (NB) cell line SK-N-AS showed that AB1 displayed potency at least equivalent to JTE-013 in affecting signaling molecules downstream of S1P<sub>2</sub>. Similarly, AB1 inhibition of the growth of SK-N-AS tumor xenografts was improved when compared to JTE-013. Cell viability assays excluded that this enhanced AB1 effect is caused by inhibition of cancer cell survival. Both JTE-013 and AB1 trended to inhibit (C-C motif) ligand 2 expression and were able to significantly inhibit subsequent tumor-associated macrophage infiltration in NB xenografts. Interestingly, AB1 was more effective than JTE-013 in inhibiting the expression of the pro-fibrotic mediator connective tissue growth factor. TUNEL assay and cleaved caspase-3 detection further demonstrated that apoptosis was increased in AB1-treated NB xenografts as compared to JTE-013. Overall, the modification of JTE-013 to produce the AB1 compound improved potency, intravenous pharmacokinetics, cellular activity, and anti-tumor activity in NB, and may have enhanced clinical and experimental applicability.
Introduction

Sphingosine-1-phosphate (S1P) is a pleiotropic lipid mediator that acts primarily by interaction with its five G protein-coupled receptors, named S1P<sub>1-5</sub>, on the cell surface (Maceyka et al., 2012). Among these, S1P<sub>1</sub> and S1P<sub>2</sub> are widely expressed in most tissues; S1P<sub>3</sub> is highly expressed in heart, lung, spleen, kidney, intestine and diaphragm; S1P<sub>4</sub> is specifically expressed in lymphoid tissues and highly expressed in blood cells while S1P<sub>5</sub> is restricted to the brain, skin and highly expressed in natural killer cells (Aarthi et al., 2011). The existing studies have shown that S1P/S1PR axis plays critical roles in a wide variety of physiological and pathophysiological processes including cancer.

S1P<sub>2</sub> was originally cloned from rat aortic smooth muscle cells in 1993 (Okazaki et al., 1993). By coupling primarily to the G<sub>12/13</sub> heterotrimeric G protein pathway, S1P<sub>2</sub> mediates different cellular functions and pathologies critical to immune, nervous, metabolic, cardiovascular, musculoskeletal, and renal systems (Adada et al., 2013). Although it is well-known that S1P<sub>2</sub> regulates the Rho/Rho kinase pathway to inhibit tumor cell migration and lymphoma development (Cattoretti et al., 2009; Muppidi et al., 2014), studies from our own group (Li et al., 2008a; Li et al., 2009a; Li et al., 2011; Li et al., 2014) as well as the others (Young et al., 2009; Ponnusamy et al., 2012; Orr Gandy et al., 2013) have found that S1P<sub>2</sub> plays important roles in tumor growth and progression in a variety of cancers, indicating that S1P<sub>2</sub> also acts as a pro-tumorigenic receptor. The latter findings suggest potential therapeutic avenues that exploit S1P<sub>2</sub> to inhibit tumor growth in some situations.

JTE-013 is a commonly used S1P<sub>2</sub> antagonist, developed by Central Pharmaceutical Research Institute, Japan Tobacco Inc. In its patent application, it stated that it inhibited the specific binding of radiolabeled S1P to membranes of CHO cells transfected with human and rat S1P<sub>2</sub>, with IC<sub>50</sub> values of 17 ± 6 nM and 22 ± 9 nM, while it did not affect S1P binding to S1P<sub>3</sub> and S1P<sub>1</sub>, at concentrations up to 10 μM (Osada et al., 2002). Based on these data, JTE-013 has been considered a specific S1P<sub>2</sub> antagonist. It has been
widely used to characterize S1P₂-dependent effects (Sanchez et al., 2007; Salomone and Waeber, 2011). However, Salomone et al. found that JTE-013 inhibited not only S1P-induced vasoconstriction, but vasoconstriction induced by KCl-, the prostanoid analog U46619- and endothelin-1 as well. This effect was also observed in S1P₂ null mice (Salomone et al., 2008), strongly suggesting that JTE-013 binds to other biological targets. In fact, Long et al. have shown that JTE-013 may also function as an S1P₄ antagonist (Long et al., 2010). In addition, our findings have shown that JTE-013 may be unstable in vivo and is rapidly metabolized in liver microsomes (Swenson et al., patent WO/2011/159864). The important role of S1P₂ in health and disease and the lack of specificity and stability of JTE-013 have driven our interest in developing more specific, potent and stable JTE-013 analogs for potential clinical and experimental use.
Materials and methods

Materials

S1P was purchased from Biomol (Plymouth Meeting, PA) and fatty-acid free BSA was from Sigma (Saint Louis, MO). JTE-013 (N-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1H-pyrazolo[3,4-b]pyridin-6-yl]-hydrazinecarboxamide) was purchased from Tocris (Ellisville, MO) and AB1 (N-(1H-4-isopropyl-1-allyl-3-methylpyrazolo[3,4-b]pyridine-6-yl)-amino-N’-(2,6-dichloropyridine-4-yl) urea) was provided by Arroyo Biosciences LLC (Princeton, NJ). Antibodies for p-AKT (#9271), Akt (#9272), p-ERK (#4370), ERK (#9102), cleaved caspase-3 (#9664) and the Jurkat apoptosis cell lysates (#2043) were from Cell Signaling Technology (Beverly, MA) while connective tissue growth factor (CTGF; sc-14939) and β-Actin (sc-8432) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Synthesis of AB1

The synthesis scheme of AB1 can be found in the patent application WO/2013/148460 (Swenson, patent WO/2013/148460). The analytical data for AB1 is as follows: 1H 300mHz NMR (CDCl3+CD3OD) 7.41 (2H, s), 6.38 (1H, s), 5.07-4.97 (2H, m), 4.77 (2H, d, J=6Hz), 3.41-3.29 (1H, m), 2.51 (3H, s), 1.24 (6H, d, J=7Hz), MS (M/z MH+) 434.2. Elemental analysis calculated C 52.4%, H 4.87%, N 22.57. Found C 52.21%, H 4.88%, N 22.53%.

FLIPR assay

The calcium flux assay on a FLIPR TETRA instrument (FLIPR assay) was performed by EMD Millipore to profile test compounds for dose-dependent agonist and antagonist activities on S1P1−5. Briefly, the agonist assay was conducted on a FLIPR TETRA instrument where the test compounds, vehicle controls, and the reference agonist S1P were added to the assay plate after a fluorescence baseline was established. Duration of 180 seconds was used to assess each compound’s ability to activate each S1PR. Upon
completion of the agonist assay, the assay plate was removed from the FLIPR TETRA and incubated at 25°C for 7 minutes. After that, the assay plate was placed back in the FLIPR TETRA and the antagonist assay was initiated. Using EC80 potency values determined during the agonist assay, all pre-incubated sample compound wells were challenged with EC80 concentration of the reference agonist S1P after establishment of a fluorescence baseline. It was also a total of 180 seconds and was used to assess each compound’s ability to inhibit each S1PR. All assay plate data were subjected to appropriate baseline corrections. After baseline corrections were applied, maximum fluorescence values were exported and data processed to calculate percentage activation (relative to Emax reference agonist S1P and vehicle control values) and percentage inhibition (relative to EC80 and vehicle control values). All dose response curves were generated using GraphPad Prism.

**Intravenous pharmacokinetic analysis**

This assay was performed by NoAb Biodiscoveries. Male CD-1 mice were used in this study. Briefly, a catheter was implanted in the carotid artery of each mouse to facilitate the subsequent repeated blood draws. Then the tested compounds were given intravenously at 1mg/kg. A small amount of blood (30μl) was taken from the catheter at each different time points and the drug concentrations in the blood were determined by the HPLC/MS analysis.

**Cell culture**

Glioblastoma (GB) cell lines U87, U118 and the neuroblastoma (NB) cell line SK-N-AS were obtained from the American Type Culture Collection. They were cultured as previously described (Lepley et al., 2005; Li et al., 2011).

**Migration assay**

Migration assay was done in 96-well chemotaxis microchamber (Neuroprobe, Gaithersburg, MD), as described previously (Li et al., 2009b). Briefly, a polycarbonate filter (8μm pore size) was coated with 50
µg/ml fibronectin. S1P was diluted and added into the lower chamber at 85 µl per well. GB cells were serum starved for 2 h prior to trypsinizing and pretreated with or without JTE-013 and AB1 for 10 min. Then they were placed in the upper compartment at 5 x 10^4 per well in 0.39 ml medium and allowed to migrate 5 h at 37°C. The filter was then fixed overnight at 4°C and the nonmigrated cells were removed with a cotton swab. Attached cells were stained with 0.1% crystal violet and eluted with 10% acetic acid in 96-well plates. The absorbance was measured at 595 nm.

**Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay**

The viability of SK-N-AS cells treated with JTE-013 or AB1 was determined by the MTT assay, as previously described (Li et al., 2013). Briefly, SK-N-AS cells were seeded in 96-well plates and treated with different concentrations of JTE-013 or AB1 for different time followed by incubation of MTT at 37°C for 2 h. The insoluble formazan formed in viable cells were dissolved by DMSO (Sigma) and the absorbance was measured at 595 nm by using Bio-Rad Microplate Reader (Model 680). Results were presented as percentage of cell viability relative to the non-drug-treated controls.

**Subcutaneous NB tumor model**

Animal experiments were conducted according to our institution's and the National Research Council's guide for the care and use of laboratory animals. Six-week-old female athymic NCr-nu/nu nude mice (National Cancer Institute, Frederick, MD) were used in this study. Briefly, each mouse received a subcutaneous flank injection containing 1 x 10^7 SK-N-AS cells in 0.1 ml PBS. After the tumor size reached around 100mm³, the mice were randomized into three groups: vehicle control, JTE-013 and AB1. Both JTE-013 and AB1 were dissolved in DMSO first, diluted with 2% (2-hydroxypropyl)-β-cyclodextrin (Sigma) in PBS and were given by gavage at 30mg/Kg daily for consecutive 14 days. Tumors were measured every other day with a caliper and tumor volumes were calculated using the following formula: TV = length X width² X 0.52. Two weeks later, the mice were euthanized and tumor masses were collected for different assays.
Western blot

Treated SK-N-AS cells or NB xenograft tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer followed by western blot analysis as previously described (Li et al., 2009b).

Quantitative real-time PCR

SYBR Green-based quantitative real-time PCR was carried out as described (Li et al., 2009a). Primers were designed using Primer Express™ 2.0 (Applied Biosystems) and the detected genes were CTGF (Li et al., 2008a) and chemokine (C-C motif) ligand 2 (CCL2) (Li et al., 2014).

CCL2 ELISA

The homogenates of NB xenografts in RIPA buffer were analyzed for CCL2 production using Human CCL2 ELISA Development Kit (PeproTech, Rocky Hill, NJ) according to the manufacturer’s instructions.

F4/80 immunohistochemistry staining

The paraffin-embedded blocks from NB xenografts treated with or without JTE-013 and AB1 were cut for 5μm sections followed by F4/80 immunohistochemistry staining (MCA497R, 1:200, AbD Serotec) and quantification as previously described (Li et al., 2014).

TUNEL assay

TUNEL assay was performed on the paraffin sections using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions followed by quantification as previously described (Li et al., 2011).

Statistical Analysis

All experiments on cell lines were performed at least three times on separate occasions. Data are presented as means ± SE from representative experiments. Statistical significance of difference between
groups was determined using ANOVA followed by posttests or two-tailed homoscedastic Student’s $t$-test as specified in each figure.
Results

**AB1 vs JTE-013 as S1P2 antagonists and stability in vivo**- JTE-013 is the current literature standard S1P2 antagonist but is unstable in vivo (Swenson et al., patent WO/2011/159864). Through structural modification, a series of JTE-013 derivatives named AB compounds were synthesized and the FLIPR assay was conducted to determine their agonistic and antagonistic activities on S1P1-5. Among them, AB1 (Fig. 1) had the strongest S1P2 antagonist activity, with an IC50 of 3.5 nM versus 11 nM of JTE-013 (Fig. 2A). In contrast, no significant agonistic or antagonistic activities on other S1PRs were observed at micromolar levels (Supplemental Figure 1). Further, pharmacokinetic analysis following intravenous administration showed that the blood concentration of AB1 in mice remained higher than that of JTE-013 over 12 hours (Fig. 2B), indicating either better stability or slower clearance of AB1 in vivo. These data suggest that AB1 may have improved potency and stability in vivo.

**AB1 vs JTE-013 on glioblastoma cell migration**- A well-described biological consequence of S1P2 signal transduction is inhibition of cell migration (Lepley et al., 2005). To compare the efficiency of JTE-013 and AB1 as S1P2 antagonists, cell migration assays were performed. GB cell lines U118 and U87 were used since it is well-known that S1P inhibits cell migration in S1P2 high-expressing U118 cells while S1P induces cell migration in S1P2 low-expressing U87 cells ((Lepley et al., 2005); Fig. 3A and 3C). AB1 treatment was moderately more potent than JTE-013 in reversing S1P-mediated cell migration inhibition in U118 cells and enhancing S1P-stimulated cell migration in U87 cells via blocking S1P2 signaling (Fig. 3B and 3D).

**AB1 vs JTE-013 on the levels of molecules downstream of S1P2 in SK-N-AS cells**- S1P2 exerts diverse cellular functions by regulating different downstream effector molecules. Our prior studies as well as studies performed by others have shown that such molecules include intracellular signaling mediators (p-Akt, p-ERK), as well as growth and differentiation modulators such as CTGF (Sanchez et al., 2007; Li et al., 2008a). To further compare the efficiency of JTE-013 and AB1 in vitro, western blot analysis was
performed. Similar to JTE-013, AB1 was able to reverse S1P-induced Akt inhibition and inhibit S1P-induced ERK activation at concentrations between 100 nM and 1μM (Fig. 4A). Quantitative real-time PCR further showed that AB1 was relatively more effective than JTE-013 at inhibiting S1P-induced CTGF mRNA expression (Fig. 4B).

AB1 vs JTE-013 on SK-N-AS cell-based NB xenograft model- In our prior study, JTE-013 significantly inhibited the growth of NB xenografts (Li et al., 2011). Here we find that AB1 was again somewhat more potent than JTE-013 in inhibiting the growth of NB xenografts by both tumor size (Fig. 5A) and tumor weight at 14 days post-treatment (Fig. 5B). Taken together, the above data strongly suggest that AB1 may have enhanced in vivo anti-tumor activity compared to JTE-013.

AB1 vs JTE-013 on cell viability in SK-N-AS cells- To investigate the potential mechanisms of AB1’s tumor inhibitory effect, cell viability was assessed in SK-N-AS cells treated with JTE-013 or AB1. MTT assays showed that AB1 is less potent than JTE-013 in terms of reduced cell viability at concentrations higher than 50 μM in SK-N-AS cells, while they had similar potency at lower concentrations (Fig. 6), suggesting that the improved inhibitory effect elicited by AB1 is not caused by direct inhibition of cell survival on cancer cells.

AB1 vs JTE-013 on CCL2 expression and tumor-associated macrophage (TAM) infiltration in NB xenografts- In order to elucidate the mechanism of AB1’s enhanced anti-tumor effect, we quantified effects on the gene expression levels of several S1P2 downstream molecules in treated NB xenografts. One of such genes is CCL2 (Li et al., 2014). Expression of CCL2 has been shown to be positively correlated with TAM infiltration (Zhang et al., 2010). In our prior study, blockade of S1P2 signaling by JTE-013 not only inhibited the growth of NB xenografts (Li et al., 2011), but also reduced CCL2 expression and the subsequent TAM infiltration (Li et al., 2014), suggesting inhibition of CCL2 is beneficial to anti-cancer therapy. As expected, CCL2 expression trended to be decreased at both mRNA and protein levels in JTE-013 or AB1-treated NB xenografts (Fig. 7A and 7B). Further,
immunohistochemical staining for the murine macrophage marker F4/80 showed that both JTE-013 and AB1 significantly inhibited the TAM infiltration. However, AB1 did not display any improved inhibitory effects (Fig. 7C), indicating that the enhanced anti-tumor effect of AB1 was not due to effects on CCL2 expression and subsequent TAM infiltration.

**AB1 vs JTE-013 on tumor fibrosis and apoptosis in NB xenografts**- CTGF is a central mediator of fibrosis (Lipson et al., 2012). Interestingly, AB1 inhibited CTGF expression to a greater extent than JTE-013 at both the mRNA and protein levels (Fig. 8A and 8B), suggesting that the improved anti-tumor effect of AB1 may be partially due to its beneficial effects on tumor fibrosis. Histological studies utilizing Ki67 staining did not find any significant difference in number of Ki67-positive proliferating cells among three groups (Supplemental Figure 2). However, TUNEL staining (Fig. 9A) and cleaved caspase-3 detection (Fig. 9B) showed that AB1 was more potent than JTE-013 at inducing tumor cell apoptosis on these NB xenografts. Taken together, our data suggest that the improved anti-tumor effect elicited by AB1 may be attributed to effects on tumor fibrosis and tumor apoptosis in NB xenografts.
Discussion

NB is the most common extra-cranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy. It has a broad spectrum of clinical behavior which can range from spontaneous regression to dissemination and death (Rossler et al., 2008). Treatment and survival for children with NB have been greatly advanced by multimodal treatment protocols driven by collaborative groups such as the Children’s Oncology Group. Unfortunately, during the last decade treatment intensification strategies have failed to improve survival, while treatment-related morbidity has increased (Maris et al., 2007). These trends underscore the need for novel treatment approaches in NB.

We have previously shown that S1P1, S1P2 and S1P3 are abundantly expressed in NB and that S1P2 is responsible for S1P-induced VEGF expression in NB (Li et al., 2011). VEGF is a key mediator of angiogenesis, which is a prerequisite for tumor growth and metastasis. We have also demonstrated that S1P2 mediates S1P-induced CCL2 expression and the subsequent infiltration of pro-tumor and pro-metastatic TAMs in NB (Li et al., 2014). Furthermore, blockage of S1P2 signaling in NB significantly inhibited tumor growth (Li et al., 2011). All the above findings strongly support a cancer-promoting role for S1P2 in NB and suggest that the development of S1P2 as an anti-cancer target in NB is a viable strategy. Of note, although recent findings have shown that genetic loss or mutation of S1P2 results in the development of diffuse large B-cell lymphoma in mice and humans (Cattoretti et al., 2009; Muppidi et al., 2014), complete suppression of S1P2 activity is unlikely to be achieved by pharmacologic intervention and would not be expected to promote lymphoma in humans.

Given the above findings we pursued the development of JTE-013 derivatives in order to improve on the known performance deficiencies of JTE-013. The AB compounds presented herein are based on a modification of two structural motifs in the JTE-013 parent compound that were predicted to improve its potency and in vivo stability. Subsequent activity and pharmacological screens demonstrated that AB1 was highly active and stable in the circulation, prompting its further testing as a novel S1P2 antagonist.
Interestingly, while tumor cell migration and effects on S1P-induced CTGF expression were consistent with an enhanced potency of AB1 over JTE-013 in vitro, we found no distinguishable difference on S1P-mediated Akt and ERK phosphorylation in NB cells. However, in addition to S1P₂, Akt and ERK are also downstream effectors of S1P₁ and S1P₃, which are also abundantly expressed in NB (Li et al., 2011) and may obscure effects on these markers in cell-based assays. Further testing in murine xenograft models demonstrated that AB1 treatment was somewhat more effective at halting tumor growth, which we determined was not due to direct inhibition of cell survival on cancer cells or quantifiable differences in the decreases in CCL2 expression or subsequent TAM infiltration in NB xenografts, despite AB1’s increased activity and stability.

Additional investigation revealed lower CTGF levels and more apoptotic cells in AB1-treated NB xenografts compared to those of JTE-013, suggesting that AB1’s enhanced anti-tumor activity is a consequence of its blocking S1P-S1P₂ effects on tumor fibrosis and tumor cell apoptosis. In our previous studies we demonstrated that S1P₂ controlled CTGF expression in Wilms tumor, another pediatric solid malignancy, resulting in anti-proliferative effects on cancer cell itself (Li et al., 2008a). However, CTGF is known to play a significant role in tumor cell “epithelial to mesenchymal transition”, a well-understood process that underlies fibrosis, and can facilitate metastasis in the context of the tumor microenvironment (Singh and Settleman, 2010). Additionally, CTGF promotes desmoplastic reactions in tumors that resemble fibropoliferative disease and serve to shield tumors from immune surveillance or even chemotherapy (Bennewith et al., 2009). S1P-S1P₂ interaction is well-known to increase extracellular matrix deposition, again contributing to scar formation (Sobel et al., 2013). Furthermore, the ability of CTGF to promote tumor angiogenesis and protect tumor cells from hypoxia-induced apoptosis (Chu et al., 2008) likely contributes to the increased tumor cell death in AB1-treated NB tumors in our study. Finally, S1P₂ signaling has been shown to reduce apoptosis and promote survival of normal and tumor cells in response to a number of pro-apoptotic triggers such as toxic chemotherapeutics (Donati et al., 2007; Li et
al., 2008b; Frias et al., 2010) and ischemia-reperfusion injury (Kang and Lee, 2014) among others (Donati et al., 2007), further supporting the utility of AB1 as an anti-tumor therapy.

In summary, here we report that the novel modification of the S1P$_2$ antagonist JTE-013 to produce AB1. AB1 has moderately improved potency and intravenous pharmacokinetics that demonstrate better stability. In the context of NB it also appears to have better cellular activity and anti-tumor activity. Based on the findings we conclude that AB1 may have enhanced clinical and experimental applicability, overcoming some of the shortcomings of JTE-013.
Acknowledgements

We thank Dr. Flavia Pereira for her help on the statistical analysis.
Authorship Contributions:

Participated in research design: Li, Swenson, Hla, Shapiro, and Ferrer

Conducted experiments: Li, and Jana

Contributed new reagents or analytic tools: Swenson, Jana, and Stolarzewicz

Performed data analysis: Li, and Swenson

Wrote or contributed to the writing of the manuscript: Li, Swenson, Harel, Hla, Shapiro, and Ferrer.
References


Footnotes

a) This work was supported by the National Institutes of Health [grants R01CA168903, PO1 CA77839], the Seraph Foundation, the Burr Curtis Surgical Endowment, and Arroyo BioSciences, LLC.

b) Patent information:


Figure legends

FIGURE 1. The chemical structure of AB1.

FIGURE 2. The biological characteristic of JTE-013 and AB1. (A) FLIPR assay was conducted to profile JTE-013 and AB1 for dose-dependent agonist and antagonist activities on S1P_1-5. The result for S1P_2 antagonist activity was shown here. (B) Intravenous pharmacokinetics of JTE-013 and AB1 (1mg/Kg) in mice (n=3 per group). #, \( P < 0.05 \), ##, \( P < 0.01 \) versus corresponding JTE-013-treated, using two-tailed homoscedastic Student’s \( t \)-test.

FIGURE 3. Effect of JTE-013 and AB1 on GB cell migration. (A and C) Migration assays were done in GB cell lines U118 (A) and U87 (C) using S1P at the indicated concentrations. *, \( P < 0.05 \), **, \( P < 0.01 \), ***, \( P < 0.001 \) versus vehicle control, using one-way ANOVA followed by Bonferroni multiple comparison test. (B and D) U118 (B) and U87 (D) cells were pretreated with different concentrations of JTE-013 and AB1 for 10 min followed by the migration assays using 100 nM of S1P as a stimulator. **, \( P < 0.01 \), ***, \( P < 0.001 \) versus S1P alone; ##, \( P < 0.01 \), ###, \( P < 0.001 \) versus corresponding JTE-013-treated, using two-way ANOVA followed by Bonferroni posttests.

FIGURE 4. Effect of JTE-013 and AB1 on S1P_2 signaling pathway in SK-N-AS cells. (A) Cells were pretreated with JTE-013 or AB1 for 30min and stimulated with 100 nM of S1P for 10 min followed by western blot analysis for the indicated molecules. (B) Cells were pretreated with JTE-013 or AB1 for 30min and stimulated with 100 nM of S1P for 2 h followed by quantitative real-time PCR. ***, \( P < 0.001 \) versus S1P alone, using two-way ANOVA followed by Bonferroni posttests.

FIGURE 5. Effect of JTE-013 and AB1 on NB xenografts. (A) The tumor growth curve of mice treated with vehicle control (n=10), JTE-013 (n=5) and AB1 (n=5) by gavage at 30mg/Kg daily for 14 days. *, \( P < 0.05 \), ***, \( P < 0.001 \) versus corresponding control, using two-way ANOVA followed by Bonferroni posttests. (B) The tumor weight of three groups when the mice were euthanized. Data are mean±SE . *, \( P < 0.05 \) versus control, using one-way ANOVA followed by Bonferroni multiple comparison test.
FIGURE 6. Effect of JTE-013 and AB1 on cell viability in SK-N-AS cells. Cells were plated to 96-well plates. The next day they were treated with JTE-013 and AB1 for 1-3 days followed by the MTT assay. Percentage of cell viability was calculated relative to the DMSO control. Data are the mean± SE of triplicates. *, P < 0.05, **, P<0.01 versus corresponding JTE-013-treated, using two-tailed homoscedastic Student’s t-test.

FIGURE 7. Effect of JTE-013 and AB1 on CCL2 expression and TAM infiltration in NB xenografts. (A and B) Quantitative real-time PCR (A) and CCL2 ELISA (B) were performed to detect the CCL2 mRNA and protein expression in NB xenografts treated with/ without JTE-013 and AB1. (C) Immunohistochemistry staining was performed to detect the F4/80 positive cells in JTE-013/ AB1-treated NB xenografts and photos were taken under the light microscope (20x). A representative picture from each group (right) and the quantitative result were shown. *, P < 0.05, **, P < 0.01 versus control, using one-way ANOVA followed by Bonferroni multiple comparison test.

FIGURE 8. Effect of JTE-013 and AB1 on CTGF expression in NB xenografts. (A) Quantitative real-time PCR was performed to detect the CTGF mRNA expression in NB xenografts treated with/ without JTE-013 and AB1. *, P < 0.05 versus control, using one-way ANOVA followed by Dunnett’s multiple comparison test. (B) Western blot analysis was performed to detect the CTGF protein expression in these treated NB xenografts. CTGF band densities were quantified by the ImageJ. *, P < 0.05 versus control. Instead of using one-way ANOVA, two-tailed homoscedastic Student’s t-test was used here to compare AB1 group and control group due to the big variation of JTE-013 group.

FIGURE 9. Effect of JTE-013 and AB1 on tumor apoptosis in NB xenografts. (A) TUNEL staining was performed to detect the apoptotic cells on NB xenografts treated with/ without JTE-013 and AB1 and photos were taken under the light microscope (20x). A representative picture from each group and the quantitative result were shown. (B) Western blot analysis was performed to detect the cleaved caspase-3 bands in the homogenates of NB xenografts treated with/ without JTE-013 and AB1. Jurkat cell lysates
untreated or treated with etoposide were served as negative and positive controls for cleaved caspase-3 bands. *, $P < 0.05$ versus control, using one-way ANOVA followed by Dunnett’s multiple comparison test.
Figure 1
Figure 2

A) S1P2 antagonist activity

B) Pharmacokinetics

Normalized Percentage Activation

Log [Concentration], M

Blood Drug Concentration (ng/ml)

Time (h)

JTE-013
AB1

# p = 0.053
Figure 3
Figure 4
Figure 5

A  Tumor Growth

B  Tumor Weight

- control
- JTE-013
- AB1

Tumor Volume (mm$^3$)

Tumor Weight (g)

Days

Figure 5
Figure 6
Figure 7
Figure 8
Figure 9