Antitumor Activity of a Novel Sphingosine-1-Phosphate 2 Antagonist, AB1, in Neuroblastoma[§]

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ABSTRACT

The bioactive lipid sphingosine-1-phosphate (S1P) and its receptors (S1P_{1–5}) play critical roles in many pathologic processes, including cancer. The S1P axis has become a bona fide therapeutic target in cancer. JTE-013 [*N*-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-hydrazinecarboxamide], a known S1P₂ antagonist, suffers from instability in vivo. Structurally modified, more potent, and stable S1P₂ inhibitors would be desirable pharmacological tools. One of the JTE-013 derivatives, AB1 [*N*-(1*H*-4-isopropyl-1-allyl-3-methylpyrazolo[3,4-*b*]pyridine-6-yl)-amino-*N'*-(2,6-dichloropyridine-4-yl) urea], exhibited improved S1P₂ antagonism compared with JTE-013. Intravenous pharmacokinetics indicated enhanced stability or slower clearance of AB1 in vivo. Migration assays in glioblastoma showed that AB1 was slightly more effective than JTE-013 in blocking S1P₂-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 S1P₂. Similarly, AB1 inhibition of the growth of SK-N-AS tumor xenografts was improved compared with 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 profibrotic mediator connective tissue growth factor. The terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling assay and cleaved caspase-3 detection further demonstrated that apoptosis was increased in AB1-treated NB xenografts compared with JTE-013. Overall, the modification of JTE-013 to produce the AB1 compound improved potency, intravenous pharmacokinetics, cellular activity, and antitumor activity in NB and may have enhanced clinical and experimental applicability.

Introduction

Sphingosine-1-phosphate (S1P) is a pleiotropic lipid mediator that acts primarily through interaction with its five G protein-coupled receptors, named S1P_{1–5}, on the cell surface (Maceyka et al., 2012). Among these, S1P₁ and S1P₂ are widely expressed in most tissues; S1P₃ is highly expressed in the heart, lung, spleen, kidney, intestine, and diaphragm; S1P₄ is specifically expressed in lymphoid tissues and is highly expressed in blood cells, whereas S1P₅ is restricted to

the brain and skin and is highly expressed in natural killer cells (Aarthi et al., 2011). Existing studies have shown that the S1P/sphingosine-1-phosphate receptor (S1PR) axis plays critical roles in a wide variety of physiologic and pathophysiological processes, including cancer.

S1P₂ was originally cloned from rat aortic smooth muscle cells in 1993 (Okazaki et al., 1993). By coupling primarily to the G_{12/13} heterotrimeric G protein pathway, S1P₂ 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 S1P₂ 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 group (Li et al., 2008a, 2009a, 2011, 2014) as well as others (Young et al., 2009; Ponnusamy et al., 2012; Orr Gandy et al.,

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ABBREVIATIONS: AB1, *N*-(1*H*-4-isopropyl-1-allyl-3-methylpyrazolo[3,4-*b*]pyridine-6-yl)-amino-*N'*-(2,6-dichloropyridine-4-yl) urea; CCL2, chemokine (C-C motif) ligand 2; CTGF, connective tissue growth factor; ERK, extracellular signal-regulated kinase; FLIPR, fluorescent imaging plate reader; GB, glioblastoma; JTE-013, *N*-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-hydrazinecarboxamide; MTT, methylthiazolylidiphenyl-tetrazolium bromide; NB, neuroblastoma; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor; TAM, tumor-associated macrophage; U46619, (5*Z*)-7-[(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid.

Fig. 1. The chemical structure of AB1.

2013) have found that S1P₂ plays important roles in tumor growth and progression in a variety of cancers, indicating that S1P₂ also acts as a protumorigenic receptor. The latter findings suggest potential therapeutic avenues that exploit S1P₂ to inhibit tumor growth in some situations.

JTE-013 [*N*-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-hydrazinecarboxamide] is a commonly used S1P₂ antagonist, developed by the Central Pharmaceutical Research Institute of Japan Tobacco, Inc. Its patent application stated that JTE-013 inhibited the specific binding of radiolabeled S1P to membranes of Chinese hamster ovary cells transfected with human and rat S1P₂, with IC₅₀ values of 17 ± 6 nM and 22 ± 9 nM, whereas it did not affect S1P binding to S1P₃ and S1P₁ at concentrations up to 10 μM (Osada et al., 2002). On the basis of these data, JTE-013 has been considered a specific S1P₂ antagonist. JTE-013 has been widely used to characterize S1P₂-dependent effects (Sanchez et al., 2007; Salomone and Waeber, 2011). However, Salomone et al. (2008) found that JTE-013 inhibited not only S1P-induced vasoconstriction but also vasoconstriction induced by KCl, the prostanoid analog U46619 [(5*Z*)-7-[(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid], and endothelin-1. This effect was also observed in S1P₂ null mice (Salomone et al., 2008), strongly suggesting that JTE-013 binds to other biologic targets. In fact, Long et al. (2010) have shown that JTE-013 may also function as an S1P₄ antagonist. In addition, our findings have shown that JTE-013 may be unstable in vivo and is rapidly metabolized in liver microsomes (Swenson et al., 2011). Our interest in developing more specific, potent, and stable JTE-013 analogs for potential clinical and experimental use is driven by the important role of S1P₂ in health and disease and the lack of data on the specificity and stability of JTE-013.

Materials and Methods

S1P was purchased from Biomol (Plymouth Meeting, PA) and fatty acid free bovine serum albumin was from Sigma-Aldrich (St. Louis, MO). JTE-013 was purchased from Tocris (Ellisville, MO) and AB1 [*N*-(1*H*-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 Akt, p-Akt, extracellular signal-regulated kinase (ERK), p-ERK, cleaved caspase-3, and the Jurkat apoptosis cell lysates were from Cell Signaling Technology (Beverly, MA), whereas connective tissue growth factor (CTGF) and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Synthesis of AB1. The synthesis scheme of AB1 can be found in the patent application (Swenson, 2013). The analytical data for AB1 are as follows: 1*H* 300 MHz nuclear magnetic resonance (CDCl₃ + CD₃OD), 7.41 s (2*H*), 6.38 s (1*H*), 5.07 to 4.97 m (2*H*), 4.77 d (2*H*, *J* = 6 Hz), 3.41 to 3.29 m (1*H*), 2.51 s (3*H*), 1.24 d (6*H*, *J* = 7 Hz), and MS (*m/z* MH⁺) 434.2. Elemental analysis calculated the following: C, 52.4%; H, 4.87%; and N, 22.57 (found C, 52.21%; H, 4.88%; and N, 22.53%).

Fluorescent Imaging Plate Reader Assay. The calcium flux assay on a FLIPR^{TETRA} instrument [fluorescent imaging plate reader (FLIPR) assay] was performed by EMD Millipore (St. Charles, MO) 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, in which the test compounds, vehicle controls, and the reference agonist S1P were added to the assay plate after a fluorescence baseline was established. A 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} instrument and incubated at 25°C for 7 minutes. After that, the assay plate was placed back in the FLIPR^{TETRA} instrument and the antagonist assay was initiated. Using EC₈₀ potency values determined during the agonist assay, all preincubated sample compound wells were challenged with EC₈₀ concentration of the reference agonist S1P after establishment of a fluorescence baseline. Another duration of 180 seconds 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 were processed to calculate the percentage of activation (relative to *E*_{max} reference agonist S1P and vehicle control values) and the percentage of inhibition (relative to EC₈₀ and vehicle control values). All dose-response curves were generated using GraphPad Prism software (GraphPad Software, La Jolla, CA).

Intravenous Pharmacokinetic Analysis. This assay was performed by NoAb Biodiscoveries (Mississauga, ON, Canada). 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 1 mg/kg. A small amount of blood (30 μl) was taken from the catheter at different time points and the drug concentrations in the blood were determined by high-performance liquid chromatography analysis/mass spectrometry.

Fig. 2. The biologic characteristics of JTE-013 and AB1. (A) A FLIPR assay was conducted to profile JTE-013 and AB1 for dose-dependent agonist and antagonist activities on S1P₁₋₅. The results for S1P₂ antagonist activity are shown here. (B) Intravenous pharmacokinetics of JTE-013 and AB1 (1 mg/kg) in mice (*n* = 3 per group). #*P* < 0.05; ##*P* < 0.01 versus corresponding JTE-013-treated mice, using a two-tailed homoscedastic *t* test.

Fig. 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 analysis of variance (ANOVA), followed by the 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 minutes, followed by the migration assays using 100 nM 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 post tests. Con, concentration.

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

Migration Assay. The migration assay was performed in a 96-well chemotaxis microchamber (Neuroprobe, Gaithersburg, MD), as described previously (Li et al., 2009b). Briefly, a polycarbonate filter (8- μ m pore size) was coated with 50 μ g/ml fibronectin. S1P was diluted and added into the lower chamber at 85 μ l per well. GB cells were serum starved for 2 hours prior to trypsinization and were pretreated with or without JTE-013 and AB1 for 10 minutes. They were then placed in the upper compartment at 5×10^4 cells per well in 0.39 ml medium and allowed to migrate 5 hours 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 Assay. The viability of SK-N-AS cells treated with JTE-013 or AB1 was determined by the methylthiazolyldiphenyl-tetrazolium bromide (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 times, followed by incubation of MTT at 37°C for 2 hours. The insoluble formazan formed in viable cells were dissolved by dimethylsulfoxide (Sigma-Aldrich) and the absorbance was measured at 595 nm by using a Bio-Rad Microplate Reader (model 680; Bio-Rad Laboratories, Hercules, CA). Results are presented as the percentage of cell viability relative to the nondrug-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 1×10^7 SK-N-AS cells in 0.1 ml phosphate-buffered saline. After the tumor size reached approximately 100 mm³, the mice were randomized into three groups: vehicle control, JTE-013, and AB1. Both JTE-013 and AB1 were dissolved in dimethylsulfoxide first, diluted with 2% (2-hydroxypropyl)- β -cyclodextrin (Sigma-Aldrich) in phosphate-buffered saline, and given by gavage at 30 mg/kg daily for 14 consecutive days. Tumors were measured every other day with a caliper, and tumor volumes were calculated using the following formula: tumor volume = length $\times$ width² $\times$ 0.52. Two weeks later, the mice were euthanized and tumor masses were collected for different assays.

Fig. 4. Effect of JTE-013 and AB1 on the S1P₂ signaling pathway in SK-N-AS cells. (A) Cells were pretreated with JTE-013 or AB1 for 30 minutes and stimulated with 100 nM S1P for 10 minutes, followed by Western blot analysis for the indicated molecules. (B) Cells were pretreated with JTE-013 or AB1 for 30 minutes and stimulated with 100 nM S1P for 2 hours, followed by qPCR. *** $P < 0.001$ versus S1P alone, using two-way analysis of variance, followed by Bonferroni post tests. qPCR, quantitative real-time polymerase chain reaction.

Fig. 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 30 mg/kg daily for 14 days. * $P < 0.05$; *** $P < 0.001$ versus corresponding control, using two-way analysis of variance (ANOVA), followed by Bonferroni post tests. (B) The tumor weight of the three groups when the mice were euthanized. Data are means $\pm$ S.E. * $P < 0.05$ versus control, using one-way ANOVA, followed by the Bonferroni multiple comparison test.

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

Quantitative Real-Time Polymerase Chain Reaction. A SYBR Green-based quantitative real-time polymerase chain reaction was carried out as described (Li et al., 2009a). Primers were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) and the detected genes were CTGF (Li et al., 2008a) and chemokine (C-C motif) ligand 2 (CCL2) (Li et al., 2014).

CCL2 Enzyme-Linked Immunosorbent Assay. The homogenates of NB xenografts in radioimmunoprecipitation assay buffer were analyzed for CCL2 production using the 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 5- μ m sections, followed by F4/80 immunohistochemistry staining (MCA497R, 1:200; AbD Serotec, Oxford, UK) and quantification as previously described (Li et al., 2014).

Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-Deoxyuridine Nick-End Labeling Assay. A terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling assay was performed on the paraffin sections using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) 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 $\pm$ S.E. from representative experiments. Statistical significance of differences between groups was determined using analysis of variance, followed by post tests or the two-tailed homoscedastic t test as specified in each figure.

Results

AB1 versus 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., 2011). 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 versus 11 nM for JTE-013 (Fig. 2A). By contrast, no significant agonistic or antagonistic activities on other S1PRs were observed at micromolar levels (Supplemental Fig. 1). Furthermore, pharmacokinetic analysis after 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 versus JTE-013 on GB Cell Migration. A well described biologic 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, whereas S1P induces cell migration in S1P₂ low-expressing U87 cells (Lepley et al., 2005; Fig. 3, A and C). 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. 3, B and D).

Fig. 6. Effect of JTE-013 and AB1 on cell viability in SK-N-AS cells. Cells were plated in 96-well plates. The next day, they were treated with JTE-013 and AB1 for 1–3 days, followed by the MTT assay. The percentage of cell viability was calculated relative to the dimethylsulfoxide control. Data are the means $\pm$ S.E. of triplicates. * $P < 0.05$; ** $P < 0.01$ versus corresponding JTE-013-treated cells, using the two-tailed homoscedastic t test. Con, concentration.

Fig. 7. Effect of JTE-013 and AB1 on CCL2 expression and TAM infiltration in NB xenografts. (A and B) qPCR (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 a light microscope. A representative picture from each group (right) and the quantitative result are shown. * $P < 0.05$; ** $P < 0.01$ versus control, using one-way analysis of variance, followed by the Bonferroni multiple comparison test. Original magnification, 20 $\times$. ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative real-time polymerase chain reaction.

AB1 versus 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 1 μ M (Fig. 4A). The quantitative real-time polymerase chain reaction further showed that AB1 was relatively more effective than JTE-013 at inhibiting S1P-induced CTGF mRNA expression (Fig. 4B).

AB1 versus JTE-013 on a 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 after treatment (Fig. 5B). Taken together, the above data strongly suggest that AB1 may have enhanced in vivo antitumor activity compared with JTE-013.

AB1 versus 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 μ M in SK-N-AS cells, whereas it 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 versus JTE-013 on CCL2 Expression and Tumor-Associated Macrophage Infiltration in NB Xenografts. To elucidate the mechanism of AB1's enhanced antitumor

effect, we quantified effects on the gene expression levels of several S1P₂ downstream molecules in treated NB xenografts. CCL2 is one of these genes (Li et al., 2014). Expression of CCL2 has been shown to be positively correlated with tumor-associated macrophage (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

Fig. 8. Effect of JTE-013 and AB1 on CTGF expression in NB xenografts. (A) qPCR 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 analysis of variance (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 ImageJ software (NIH, Bethesda, MD). * $P < 0.05$ versus control. Instead of using one-way ANOVA, the two-tailed homoscedastic t test was used here to compare the AB1 group and the control group because of the large variation in the JTE-013 group.

(Li et al., 2011), but it also reduced CCL2 expression and the subsequent TAM infiltration (Li et al., 2014), suggesting that inhibition of CCL2 is beneficial to anticancer therapy. As expected, CCL2 expression tended to be decreased at both mRNA and protein levels in JTE-013-treated or AB1-treated NB xenografts (Fig. 7, A and B). Furthermore, 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 antitumor effect of AB1 was not attributable to effects on CCL2 expression and subsequent TAM infiltration.

AB1 versus 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. 8), suggesting that the improved antitumor effect of AB1 may be partially a result of its beneficial effects on tumor fibrosis. Histologic studies utilizing Ki67 staining did not find any significant difference in the number of Ki67-positive proliferating cells among the three groups (Supplemental Fig. 2). However, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling 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 antitumor effect elicited by AB1 may be attributed to effects on tumor fibrosis and tumor apoptosis in NB xenografts.

Discussion

NB is the most common extracranial solid tumor in childhood and is 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 (Rössler et al., 2008). Treatment and survival for children with NB has 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, whereas 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 vascular endothelial growth factor expression in

Fig. 9. Effect of JTE-013 and AB1 on tumor apoptosis in NB xenografts. (A) Terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling staining was performed to detect the apoptotic cells on NB xenografts treated with/without JTE-013 and AB1 and photos were taken under a light microscope. A representative picture from each group and the quantitative result are 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 served as negative and positive controls for cleaved caspase-3 bands. * $P < 0.05$ versus control, using one-way analysis of variance, followed by Dunnett's multiple comparison test. Original magnification, 20 $\times$.

NB (Li et al., 2011). Vascular endothelial growth factor 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 protumor and prometastatic TAMs in NB (Li et al., 2014). Furthermore, blockage of S1P₂ signaling in NB significantly inhibited tumor growth (Li et al., 2011). All of the above findings strongly support a cancer-promoting role for S1P₂ in NB and suggest that the development of S1P₂ as an anticancer 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 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, although 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 with those of JTE-013, suggesting that AB1's enhanced antitumor 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 antiproliferative effects on the 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). In addition, CTGF promotes desmoplastic reactions in tumors that resemble fibroproliferative 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 proapoptotic 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 antitumor therapy.

In summary, here we report the novel modification of the S1P₂ 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 antitumor activity. On the basis of these findings, we conclude that AB1 may have enhanced clinical and experimental applicability, overcoming some of the shortcomings of JTE-013.

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Authorship Contributions

Participated in research design: Li, Swenson, Hla, Shapiro, Ferrer. *Conducted experiments:* Li, Jana.

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

Performed data analysis: Li, Swenson.

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

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