Note

Synthesis and Biological Evaluation of PF-543 Derivative Containing Aliphatic Side Chain

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The PF-543 is known as a potent and selective inhibitor of sphingosine kinase (SK) 1 amongst all the SK inhibitors known to date. In a recently reported study by Pfizer on the synthesis of PF-543 derivatives and the SK inhibitory effects, the introduction of propyl moiety into sulfonyl group of PF-543 in the case of 26b revealed an excellent result of 1.7 nM of IC50 of SK1, suggesting the potential substitution of chain structure for benzenesulfonyl structure. In the present work, we aimed for identification of antitumor activity and inhibitory effects of PF-543 derivative containing aliphatic long chain (similar to known SK inhibitors) on SK1.

The synthesized compound 2 exhibited an inhibitory effect on SK1 in a manner similar to that of PF-543; the PF-543 derivative manifested similar antitumor activity on HT29, HCT116 (colorectal cancer cell line), and AGS (gastric cancer cell line) cells. Also, from the docking study conducted with PF-543 and compound 2, it was apparent that the aliphatic chain in compound 2 could probably replace benzenesulfonyl structure of PF-543.

Key words PF-543; anticancer; sphingosine kinase; derivative

Introduction

Sphingolipids have been widely studied in various disciplines and sphingosine, ceramide, and glycolipid have been exploited for their application in the field of medicine based on their importance.1,2) However, despite immense efforts, development of medicines using sphingolipid encounters difficulties due to the aspects of solubility and stability thereof. FTY720 (Fingolimod, Gilenya, Novartis), belongs to the family of sphingolipids and is the first oral therapeutic agent approved by U.S. Food and Drug Administration (FDA) for the treatment of multiple sclerosis.3) FTY720 functions as a modulator of the sphingosine-1-phosphate (SIP) receptor. Besides, FTY720 has been known to suppress sphingosine kinase (SK) 1 and exhibit antitumor activity on various cancers by activating protein phosphatase 2A (PP2A).4,5) Despite sphingolipid acts on diverse targets, Gilenya of Novartis marked the first rank sales amounting to 3185 million USD in 2017; this suggests the possibility of the development of medicines using various sphingolipids. SK transforms sphingosine in cells into SIP that promotes cell growth. In fact, patients suffering from cancer show an increase in the level of SIP.6) Besides, diverse animal experiments have revealed a reduction in the SIP level and cessation or disturbance in the growth of cancer cells by the suppression of SK. Thereby, continuous efforts have been put on the development of new antitumor agents through the development of SK inhibitors. SK has two isotypes, SK1 and SK2, and suppression of both the isotypes would lead to a reduction in the level of SIP and suppression of growth of cancer cells. Both SK1 and SK2 act in different positions in cells; however, the differences between the two roles are yet to be clarified.7) SK inhibitors initially have been developed based on the structure of sphingosine or FTY720.6) ROME (Fig. 1), which is a representative derivative of FTY720, has stereoselectively introduced methoxy group into the head group.8) ROME inhibits SK2 selectively and has been reported to exhibit anticancer effects on leukemia and breast cancer cells in animal experiments. On the contrary, the RB-005 (Fig. 1), which has a structure similar to that of FTY720, inhibits SK1 selectively.9) Synthesis of head groups with a cyclic amine structure similar to RB-005 is easier than the synthesis of the
head group of FTY720, and it has been used as a head group for various kinds of SK inhibitors. Pharmaceutical companies like Pfizer (U.S.A.), Amgen (U.S.A.), and Apogee (U.S.A.) have been continuously trying to develop the SK inhibitors of non-lipid types. In particular, the ABC294640 (Yeliva®, Fig. 1), developed by Apogee Biotec., inhibits SK2 selectively and was employed in the clinical study as an inhibitor for SK for the first time.\(^{10,11}\) ABC294640 manifested effects over prostate cancer, liver cancer, colon cancer, breast cancer, etc. in various animal experiments and the clinical trials on cases of liver cancer, liver cancer, colon cancer, breast cancer, etc. in various animal experiments and the clinical trials on cases of liver cancer, pancreatic cancer, and bile duct cancer are in progress.

PF-543 (Fig. 1) developed by Pfizer is a potent and selective animal experiment and the clinical trials on cases of liver cancer, liver cancer, colon cancer, breast cancer, etc. in various animal experiments and the clinical trials on cases of liver cancer, pancreatic cancer, and bile duct cancer are in progress. PF-543 (Fig. 1) developed by Pfizer is a potent and selective animal experiment and the clinical trials on cases of liver cancer, pancreatic cancer, and bile duct cancer are in progress. PF-543 (Fig. 1) developed by Pfizer is a potent and selective animal experiment and the clinical trials on cases of liver cancer, pancreatic cancer, and bile duct cancer are in progress. PF-543 (Fig. 1) developed by Pfizer is a potent and selective animal experiment and the clinical trials on cases of liver cancer, pancreatic cancer, and bile duct cancer are in progress. PF-543 (Fig. 1) developed by Pfizer is a potent and selective animal experiment and the clinical trials on cases of liver cancer, pancreatic cancer, and bile duct cancer are in progress. PF-543 (Fig. 1) developed by Pfizer is a potent and selective animal experiment and the clinical trials on cases of liver cancer, pancreatic cancer, and bile duct cancer are in progress.

Recently, based on the results of animal experiments employing PF-543, suppression of cancer growth with a survival rate of 100% was apparent.\(^{12}\) Such results suggest that the structure of PF-543 still can be developed as an antitumor agent. The relationship between the functional groups into the structure of PF-543 and the biological activities still remains indefinite. The benzenesulfonyl tail of PF-543 is rarely seen in other SK inhibitors. Besides, like RB-021 or compound 17 (Fig. 1), it manifested SK inhibitory effect and selectivity to SK1/SK2 commonly reduced by the introduction of benzenesulfonyl tail.\(^{9,10}\) Pfizer reported the synthesis of PF-543 derivatives in 2017 along with the results of structure–activity relationship (SAR) of the synthesized compound and SK inhibitory effect.\(^{15}\) In the paper, the synthesis of derivatives resulting from employment of various head groups and tail of PF-543 is presented. In particular, the compound 26f (Fig. 1), wherein the aminobenzimidazole group of new structure was introduced into the tail of PF-543, rendered comparatively excellent results corresponding to IC\(_{50}\) of SK1 of 0.004 \(\mu\)M PF-543 (CLP, [Caliper (fluorescein isothiocyanate (FITC)-SIP formation) enzyme assay format]. SK1 IC\(_{50}\) = 0.8 nM), which was measured through CLP. Besides, the 26a (Fig. 1), from which the methyl group of PF-543 was removed, exhibited better results than PF-543 with the IC\(_{50}\) of 0.3 nM through CLP; however, the selectivity to SK2 was reduced to half. In the case of compound 26b (Fig. 1), in which the propyl group was introduced into benzenesulfonyl group of PF-543, rendered excellent results of 1.7 nM of IC\(_{50}\) of SK1 measured through CLP, thus suggesting that the benzenesulfonyl group could be replaced with an aliphatic chain. Thereby, we intended for identification of the presence of biological activity through the introduction of the chain of the length equivalent to that of FTY720 into the tail of PF-543.

We initiated the synthesis with 3-bromo-5-methylphenol as a starting material and synthesized the PF-543 derivative (62 mg, overall yield 23%) through the 4-step synthesis process (Chart 1). With the use of 3-bromo-5-methylphenol, 1-octyn was introduced into Sonogashira coupling to synthesize the compound 3, and then the compound 4 was synthesized by hydrogenation that exploited palladium catalyst. Subsequently, compound 5 was synthesized through the coupling of compound 4 with 4-(bromomethyl)benzaldehyde and then the \((R)-(\text{−})\)-prolinol was introduced using reductive amination to synthesize the final PF-543 derivative (2). To determine whether the compound 2 has SK1 inhibitory effect similar to PF-543 (1), we investigated SK1 activity using 40 \(\mu\)M PF-543 (1) and compound 2 (Fig. 2a). The synthesized compound 2 exhibited SK1 inhibitory effects similar to that of PF-543 (1) (58 and 62%, respectively). The IC\(_{50}\) of the PF-543 and compound 2 was observed to be 196 and 10 nM, respectively (data not shown). PF-543 and compound 2 did not affect SK2 activity (Fig. 2b). The two compounds were also compared with each other for their cytotoxic effects on HT29, HCT116 (colorectal cancer cell line) and AGIS (gastric cancer cell line) cells. Compound 2 manifested the cytotoxic effects similar to that of PF-543 (Fig. 3a). From the measurements of annexin-V, the apoptotic effect of compound 2 and PF-543 were observed to be similar (Fig. 3b). In addition, we conducted the docking study using a computer to compare combinations of aliphatic chain of PF-543 derivative 2 and benzenesulfonyl tail of PF-543 (1) with SK1 (Fig. 4). From the study, the crystal structure, wherein PF-543 was coupled with SK1, was obtained from the Protein Data Bank (PDB code 4V24). The shape of coupling of compound 2 with SK1 was found to be

![Chart 1. Synthesis of PF-543 Derivative 2](image)

*Fig. 2. SK 1 and SK2 Activity of PF-543 (1) and Compound 2 (Comp-2)*

\((n = 2−3\) for each compound, results are expressed as \% of control ± standard deviation (S.D.).)*
similar to that of PF-543. The hydroxymethyl-pyrrolidine (OH and N of pyrrolidine) of compound 2 constitutes the hydrogen bonding to Asp264 and makes an electrostatic interaction (protonated amine form) with Asp264. Besides, the phenyl linker of compound 2 makes the hydrophobic interaction consisting of Ile260, Val263, Leu354, and Met358. Methylbenzene group of compound 2 is situated in between Phe259, Leu286, Leu385, and Phe389 and makes the hydrophobic interaction therein. The terminal acyl chain of compound 2, which is structurally different from PF-543, is oriented towards benzenesulfonyl of PF-543 and makes hydrophobic interactions with Leu347, Leu354, Ala360, and Phe374.

In summary, many research groups have developed SK inhibitors in the form of FTY720 or sphingosine, but PF-543, a non-lipid type SK1 inhibitor developed by Pfizer, has a unique structure and inhibits SK1 activity at low concentration. However, known SK inhibitors of the lipid type showed SK1 inhibitory effect at higher concentration than PF-543. The present study focused on the identification of SK1 inhibitory effect and comparison of antitumor activity between PF-543 and derivative by the introduction of aliphatic long chain into tail of PF-543. Compound 2 exhibited an inhibitory effect on SK1 and manifested antitumor activity in tumor cell lines in a manner similar to that of PF-543. Besides, the long chain of compound 2 maintained the direction of benzenesulfonyl of PF-543 and generated hydrophobic interaction in the docking study. PF-543 appears to be ineffective in some cancers. We believe that this problem is due to the fact that PF-543 has a non-lipid form. Compound 2 showed the possibility of substituting PF-543. We intend to explore this possibility through in vitro and in vivo studies on various cancer cells using compound 2 in the future.
Experimental

General Experimental Procedures  Reagents and solvents were purchased from commercial sources and used without purification. Flash column chromatography was performed on silica gel grade 60 (230–400 mesh). 1H- and 13C-NMR spectra were recorded on a Bruker Avance 1 spectrometer (at 400 and 100 MHz, respectively) and JEOL ECZ500R (at 500 and 125 MHz, respectively) using CDCl3 as a solvent, and chemical shift are reported in δ units. High-resolution (HR) MS data were measured on an Agilent Technologies (U.S.A.) G6520A Q-TOF mass spectrometer using electrospray ionization (ESI).

Chemical Synthesis  
3-Methyl-5-(oct-1-yn-1-yl)phenol 3

To a solution of 3-bromo-5-methylphenol (300 mg, 1.6 mmol), Pd(PPh3)4 (93 mg, 0.08 mmol), and CuI (15 mg, 0.08 mmol) in triethylamine (15 mL) was added 1-octyne (0.7 mL, 4.8 mmol) at room temperature (r.t.) After the reaction mixture was stirred at 50°C for 12 h, saturated NaH2O2 was added, and the mixture was extracted with EtOAc. The combined organic phases were washed with brine, dried, and concentrated. Flash column chromatography with n-hexane/EtOAc (5/1 (v/v)) as the eluent gave 3 (244 mg, 70%): 1H-NMR (400 MHz, CDCl3) δ: 6.71 (s, 1H), 6.65 (s, 1H), 6.55 (s, 1H), 2.34 (t, J = 7.1 Hz, 2H), 2.20 (s, 3H), 1.60–1.49 (m, 2H), 1.34–1.23 (m, 4H), 0.87 (t, J = 7.0 Hz, 3H); 13C-NMR (100 MHz, CDCl3) δ: 158.1, 141.1, 126.4, 125.8, 117.6, 117.0, 91.3, 82.4, 33.1, 30.4, 24.3, 22.7, 21.0, 15.6; ESI-HR-MS (M + H)+ /m/z calcd for C23H31O2 339.2329, found 339.2324.

3-Methyl-5-ocetylphenoxymethylbenzaldehyde 4

(3-Methyl-5-ocetylphenoxymethyl)benzaldehyde 5

To a solution of 4 (100 mg, 0.45 mmol) and K2CO3 (186 mg, 1.35 mmol) in tetrahydrofuran (THF) (15 mL) was added 4-(bromomethyl)benzaldehyde (108 mg, 0.54 mmol) at r.t. After the reaction mixture was stirred at 60°C for 12 h, (aqueous phase) was added and the mixture was extracted with EtOAc. The combined organic phases were washed with brine, dried and concentrated. Flash column chromatography with n-hexane/EtOAc (10:1 (v/v)) as the eluent gave 5 (92 mg, 60%): 1H-NMR (500 MHz, CDCl3) δ: 10.00 (s, 1H), 7.86 (d, J = 8.3 Hz, 2H), 7.55 (d, J = 8.1 Hz, 2H), 6.56 (s, 1H), 6.46 (s, 2H), 4.50 (s, 3H), 2.69–2.36 (m, 2H), 2.26 (d, J = 0.4 Hz, 2H), 1.71–1.42 (m, 2H), 1.34–1.12 (m, 10H), 0.86 (t, J = 7.0 Hz, 3H); 13C-NMR (125 MHz, CDCl3) δ: 191.7, 155.4, 144.8, 144.4, 139.5, 136.2, 130.3, 129.8, 129.2, 127.6, 121.9, 113.3, 112.4, 35.9, 32.1, 32.0, 31.4, 29.6, 29.5, 29.3, 22.8, 21.4, 14.2; ESI-HR-MS (M + H)+ /m/z calcd for C20H23O2 339.2324, found 339.2391.

(R)-(1-(4-((3-Methyl-5-octylphenoxymethyl)benzyl)phenol-2-yl)pyrrolidin-2-yl)methanol 2

To a solution of 5 (80 mg, 0.24 mmol) and (R)-(−)-prolinol (36 mg, 0.36 mmol) in 1,2-dichloroethane (10 mL) was added sodium triacetoxynorbornylate (STB) (75 mg, 0.35 mmol). After being stirred at r.t. for 12 h, the reaction mixture was diluted with water and extract with EtOAc. The extract was purified by column chromatography on silica gel with brine, dried and evaporated. The residue was washed with CH3Cl:MeOH (10:1 (v/v)) to give 62 mg (62%) of product 2: 1H-NMR (500 MHz, CDCl3) δ: 7.62 (d, J = 8.1 Hz, 2H), 7.50 (d, J = 8.1 Hz, 2H), 6.62 (s, 1H), 6.59 (s, 2H), 5.03 (s, 2H), 3.87–3.84 (m, 2H), 3.68–3.60 (m, 1H), 3.58–3.53 (m, 1H), 2.56–2.49 (m, 2H), 2.29 (s, 3H), 2.21–1.99 (m, 5H), 1.97–1.91 (m, 2H), 1.58–1.23 (m, 2H), 1.31–1.20 (m, 10H), 0.86 (t, J = 7.0 Hz, 3H); 13C-NMR (125 MHz, CDCl3) δ: 158.6, 144.7, 139.4, 131.5, 128.2, 122.4, 112.6, 112.0, 70.6, 69.1, 61.0, 60.1, 53.9, 36.1, 32.0, 31.5, 29.8, 29.6, 29.5, 25.9, 26.4, 23.5, 22.8, 21.6, 14.2; ESI-HR-MS (M + H)+ /m/z calcd for C32H42N2O2 424.3216, found 424.3291.

Sphingosine Kinase Activity Assay

Sphingosine kinase 1 and 2 inhibitory effect was measured with 40 µM PF-543 (I) and compound 2 using 100 µM sphingosine, 10 µM ATP, 0.5 ng/µL of recombinant SK1 and 0.75 ng/µL of recombinant SK2. SK1 and SK2 activity was detected with an Echelon’s Sphingosine Kinase Activity Assay kit according to the manufacturer’s protocol.

Cell Culture and Proliferation Assays

HT29, HCT116 and AGS cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified 5% CO2/95% air atmosphere. Cells were seeded in 96-well plates at a density of 3 × 104 cells/100 µL/well and incubated for 24 h. The cells were then incubated in culture medium containing synthetic compounds (20 or 40 µM). Following 24 h of incubation, the cell viability was determined using a EZ-CYTOKIT assay according to the manufacturer’s protocol.

Annexin-V Staining

Apoptosis was measured using an annexin-V FITC apoptosis detection kit, according to the manufacturer’s instructions. The cells were incubated with annexin-V-FITC and propidium iodide at room temperature. After incubation, stained cells were analyzed by Arthur™ Image Based Cell Analyzer.

Docking Study

Molecular modeling studies of compound 2 against the SK1 was performed using Schrödinger Suite 2018-1 (Schrödinger, LLC, New York, NY, U.S.A.). The PF-543 bound crystal structure of SK1 obtained from the Protein Data Bank (http://www.rcsb.org/pdb) that PDB code is 4V24. The protein preparation was revised using Protein Preparation Wizard in Maestro v.11.9. Flexible dockings were performed using the Glide v.7.8 program with standard precision method. The docking models of ligands were visualized using Discovery Studio 2018 ((Biovia, Discovery Studio Modeling Environment, Dassault Systèmes, San Diego, CA, U.S.A.).

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Conflict of Interest  The authors declare no conflict of interest.

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