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Assay to measure the secretion of sphingosine-1-phosphate from cells induced by S1P lyase inhibitors

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ABSTRACT

Inhibitors of the sphingosine-1-phosphate (S1P) degrading enzyme S1P lyase (SPL) may be useful in the therapy of inflammatory diseases by preventing lymphocyte recruitment to diseased tissues. Here we describe a cellular assay for such inhibitors, which takes advantage of the observation that a fraction of the intracellular S1P accumulated in the presence of SPL inhibitors is secreted into the medium of cultured cells. The secreted S1P is then quantified using an S1P-sensitive reporter cell line. In the routine assay protocol, human HEK293T cells are treated with SPL inhibitors in the presence of phosphatase inhibitors and sphingosine; while the phosphatase inhibitors are included to prevent the degradation of S1P secreted from the cells, sphingosine is added as source for intracellular S1P that is prone to SPL degradation. The secreted S1P in the supernatant of the cell cultures is then quantified by measuring calcium flux induced in CHO-K1 cells expressing the human $S1P_3$ receptor. Using this method SPL inhibitors were shown to induce a concentration-dependent increase of extracellular S1P under the conditions used; thus, the assay allows for the ranking of SPL inhibitors according to their potency on living cells.

1. Introduction

Sphingosine-1-phosphate (S1P) provides an essential egress signal to lymphocytes in secondary lymphoid organs, thereby initiating lymphocyte trafficking to sites of inflammation [1]. This signaling may be disrupted by inhibition of S1P lyase (SPL), which is the enzyme responsible for irreversible degradation of S1P [2]. Therefore, inhibitors of SPL have been proposed to be useful in inflammatory and autoimmune diseases by preventing lymphocyte recruitment to diseased tissues [3,4].

In the search for novel SPL inhibitors, a cellular assay is required to demonstrate SPL inhibition in living cells; this assay should serve as a tool to rank compounds according to their cellular potency. Previously, we have described an assay which monitors the increase of S1P within cells upon incubation with SPL inhibitors. The method requires extraction of the lipid from the cells, followed by S1P quantification using LC/MS [5], which obviously limits the throughput of the assay.

Here, we describe an alternative cellular assay which allows testing of SPL inhibitors in a medium-throughput format. The assay takes advantage of the observation that a fraction of the intracellular S1P – accumulated as a consequence of SPL inhibition – is secreted into the medium of cultured cells. The extracellular S1P in the culture supernatants can then be quantified without extraction by using a reporter cell line, in which S1P induces a detectable calcium flux. Thus, we provide a general method for estimation of S1P concentration in cell culture supernatants and describe its application to the assay of S1P secreted from cells after SPL inhibitor treatment.

2. Materials and methods

2.1. Cell culture

CHO-K1 cells expressing the human $S1P_3$ receptor (CHO-K1/$S1P_3$) [6] and HEK293T cells were cultured in RPMI1640 and Dubecco's Eagle medium (DMEM), respectively. Each medium contained stable glutamine and was supplemented with 10% fetal calf serum (FCS) and geneticin of which the final concentrations were 500 and 200 μg/ml, respectively. Cells were cultured at 37 °C, at 5% CO_2 and 95% relative humidity. For twice weekly passaging, cells were removed from the culture dish by washing with phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}, and incubation with 0.25% trypsin/0.1 mM EDTA for 2 min at room temperature.

Abbreviations: AM, acetoxymethylester; BSA, bovine serum albumin; FCS, fetal calf serum; FLIPR, fluorescent imaging plate reader; HBSS, Hank's buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; S1P, sphingosine-1-phosphate; Sph, sphingosine; SPL, S1P lyase.

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2.2. S1P determination via calcium flux assay

CHO-K1/S1P$_3$ cells were seeded at a density of 3500 cells/well into 384-well black plates (Corning) and incubated for 24 h. After removal of the culture medium, cells were loaded with the fluorescent dye Fluo-4 acetoxymethylester (AM) (Invitrogen) by incubation for 45 min at a concentration of 1.6 μM in 40 μl Hank's buffered salt solution (HBSS) containing 20 mM HEPES buffer, 0.1% bovine serum albumin (BSA), and 2.5 mM probenecid (Sigma). After the incubation period, cells were washed twice with 80 μl of wash buffer consisting of HBSS/HEPES and probenecid. In the second wash step, the wash buffer was removed from the wells in such a way that a final volume of 30 μl was retained. Probenecid (2.5 mM final concentration) was added to both loading and wash buffer to minimize cellular export of de-esterified Fluo-4 via intracellular anion exchange proteins.

Fifteen microliters of S1P-containing samples were added to the cells and agonist-induced calcium flux was recorded using a fluorescent imaging plate reader (FLIPR) (Molecular Devices). Samples were injected at a speed of 10 μl/s. To measure the calcium signal, cells were excited with an argon ion laser at 488 nm at 0.4–0.6 W laser power (absorption peak of Fluo-4/Ca^{2+}=494 nm, and charge-coupled device camera opening of 0.4 s). Fluorescence was recorded as outlined in the following scheme: for the baseline determination, 10 measurements every 1 s prior to the agonist injection; for the calcium signal determination after agonist injection, 60 measurements every 1 s followed by 40 measurements every 2 s. Emission was recorded by using a band spectrum filter (515–575 nm) at the emission peak of Fluo-4/Ca^{2+} (516 nm).

Kinetic data from each assay well were exported by using the FLIPR software tool (Molecular Devices). Data are shown as normalized fluorescence = $[(F_{max} - F_b)/F_b]$, where F_{max} is the maximal fluorescence obtained following agonist injection and F_b is the average baseline fluorescence before the injection of the agonist. Concentration–response curves were analyzed by the 4-parameter logistics model of the software XLfit (IDBS).

2.3. Transfection of cells with siRNA

HEK293T cells were seeded into 6-wells at a density of 250,000 cells per well. After resting for 24 h, cells were transfected with siRNA directed against human SPL (Invitrogen, Stealth™ RNAi, cat. No. SGPL1HSS113120) along with a corresponding control (Universal Control High, Invitrogen) using the Lipofectamine™ 2000 reagent (Invitrogen). Downregulation of SPL mRNA as verified using RT-PCR was typically ~90% at 48 h after transfection.

2.4. Routine protocol of cellular assay using HEK293T cells

HEK293T cells (12,000 cells/well) were seeded into 96-well plates coated with Poly L-lysine (Sigma) in 100 μl of culture medium. After resting for 24 h at 37 °C, 5% CO_2, the medium was removed and the cells were washed once with PBS. Then, 50 μl of HBSS supplemented with 1% charcoal-stripped FCS and 0.4% phosphatase inhibitor cocktail II (Sigma) were added, followed by 40 μl of the SPL inhibitors, diluted in HBSS that contained the supplements as described above, at graded concentrations in triplicates. Cells were incubated for 60 min. Thereafter, 10 μl of HBSS containing 10 μM D-erythro-sphingosine (Sph; Matreya LLC) and 4% charcoal-stripped FCS was added. Incubation was continued for further 2.5 h. Then, 80 μl of supernatants were transferred to a 96-well plate (Abgene PCR Plates; Thermo). The plate was frozen on dry ice and stored at −80 °C until analysis. After thawing in a water bath, the plate was inverted vigorously to facilitate homogenous mixing. For the quantification of S1P via calcium flux assay, 40 μl of the supernatant was transferred from the 96-well to a 384-well plate (Greiner). Samples were analyzed for S1P-induced calcium flux in CHO-K1/S1P$_3$ cells as described above. Calibration curves with S1P were run in parallel to each assay. EC_{50} values were calculated from the data using XLfit.

3. Results and discussion

3.1. Quantification of S1P via calcium flux assay

To provide a method for S1P quantification with a higher throughput than chromatograpy-based procedures, we made use of a CHO-K1 cell line that expresses the human S1P$_3$ receptor [6]. The use of S1P$_3$-expressing cells confers specificity to the detection of S1P, as this lipid is the only known natural ligand for the receptor. Titration of these cells with S1P gives rise to mobilization of intracellular calcium that can be monitored with a Ca^{2+}-sensitive dye using a FLIPR. Maximal Ca^{2+} signals were obtained with a cell density of ~2000 to 3500 cells per 384 well. Fig. 1 shows a concentration–response curve for S1P obtained with the assay, featuring EC_{50} = 3.9 nM.

Using such response curves for calibration, concentrations of S1P in cell culture supernatants were estimated. This method is more convenient than the radioreceptor-binding method for quantification of S1P [7] and complements the chromatographic methods reported earlier [8–10].

3.2. Stability of S1P in cell culture medium

Using the calcium flux assay, we determined the concentration of S1P in HBSS supplemented with 10% FCS to approximately 5 nM. Treatment of FCS with charcoal reduced the S1P concentration to undetectable levels (<1 nM).

When HEK293T cells were kept in HBSS with 10% untreated FCS for 24 h, the S1P content – as reflected by the Ca^{2+} signal in the FLIPR assay – was markedly reduced; the reduction appeared to correlate with the number of cells seeded (Fig. 2). This observation is in line with a published report [11], which concluded that phosphatases associated with the cell surface can cleave S1P. Indeed,

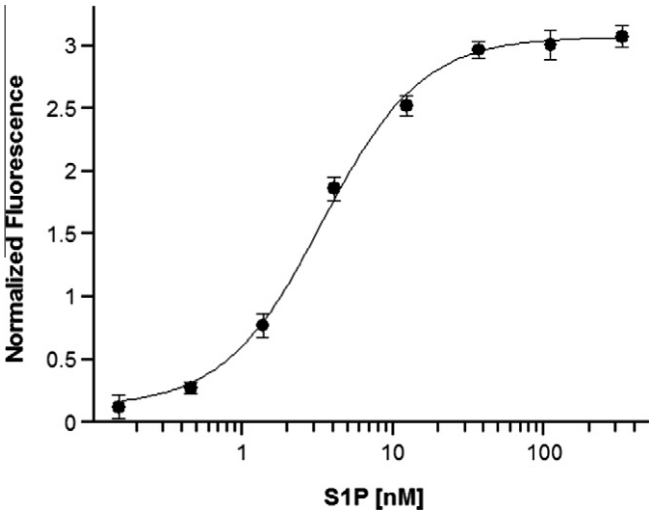

Fig. 1. Calcium mobilization induced by S1P in CHO-K1/S1P$_3$ cells. CHO-K1 cells expressing the human S1P$_3$ receptor (CHO-K1/S1P$_3$; 3500 cells per well) were seeded into 384-well plates. Twenty-four hours later, the culture medium was removed and cells were loaded with Fluo-4 AM for 60 min. Cells were washed and stimulated with graded concentrations of S1P. Ca^{2+}-dependent fluorescence was recorded before and after S1P injection using a FLIPR. The dose–response curve shows normalized fluorescence values ± standard deviation, calculated from triplicate determinations. The EC_{50} of S1P was determined to 3.9 nM.

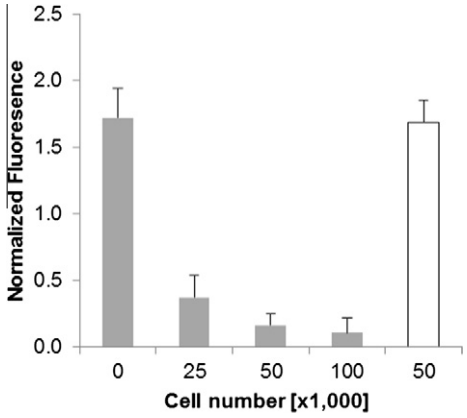

Fig. 2. Degradation of extracellular S1P by HEK293T cells in culture. Closed bars: HEK293T cells were seeded into 24-wells at the indicated numbers and kept in 1 ml HBSS containing 10% FCS (not charcoal-stripped). Open bar: HEK293T cells were seeded at a density of 50,000 cells per 24-well and kept in medium in the presence of 0.25% phosphatase inhibitor cocktail. Twenty-four hours later, the supernatants of the cell cultures were analyzed in the calcium flux assay. Data are expressed as means of normalized fluorescence values ± standard deviation of triplicates.

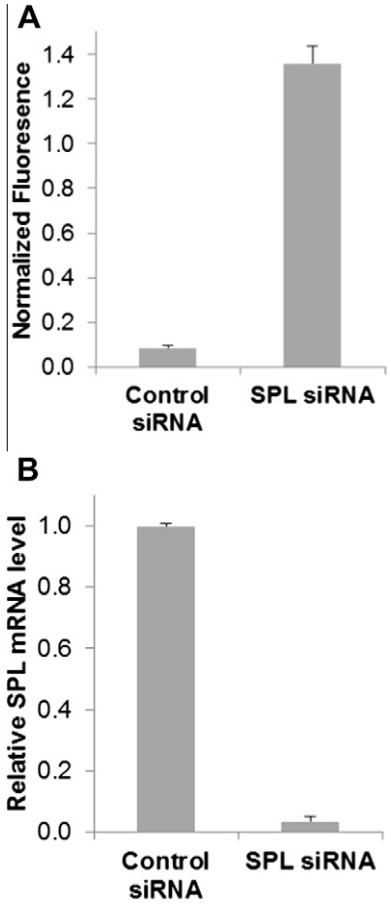

Fig. 3. Secretion of S1P from HEK293T cells transfected with SPL siRNA. (A and B) HEK293T cells were seeded at a density of 250,000 cells per 6-well and transfected with control or SPL siRNA. Forty-eight hours after transfection, the cells were analyzed for secretion of S1P in response to the siRNA treatment. Cells were cultured for 2.5 h in 1 ml HBSS medium containing 1% charcoal-stripped FCS, 0.25% phosphatase inhibitor cocktail and 1 μM Sph. Subsequently, (A) supernatants were analyzed for S1P in the calcium flux assay using CHO-K1/$S1P_3$ cells; (B) cellular SPL mRNA was quantified by RT-PCR. The data are expressed as means ± standard deviation of triplicates.

addition of phosphatase inhibitor cocktail II (Sigma) containing orthovanadate, molybdate, tartrate, and imidazole completely prevented the degradation of S1P by the cells (Fig. 2). Thus, the combined use of charcoal-stripped FCS in the medium to eliminate background S1P and the addition of phosphatase inhibitors to prevent degradation of S1P allows monitoring the secretion of S1P by HEK293T cells.

As for the amount of phosphatase inhibitor cocktail II, a final concentration of 0.25% was found to be fully effective in blocking S1P degradation without inducing cell toxicity during the course of the routine assay procedure.

3.3. Secretion of S1P from cells transfected with SPL siRNA

HEK293T cells did not secrete detectable levels of S1P when cultured in HBSS supplemented with 1% charcoal-stripped FCS and phosphatase inhibitors for 24 h. Also, when cells were incubated in the presence of 1 μM Sph to increase the intracellular pool of S1P [5], secretion of S1P into the medium was not detectable. However, when cells were transfected with siRNA directed against human SPL, an increase of extracellular S1P was observed as indicated by the calcium flux assay on CHO-K1/$S1P_3$ cells (Fig. 3A); the increase of S1P was associated with a decrease of SPL transcripts (Fig. 3B). Thus, knock-down of SPL not only causes an increase in intracellular concentrations of S1P (as previously shown [5]), but gives also rise to secretion of S1P into the culture medium.

3.4. Secretion of S1P from HEK293T cells treated with SPL inhibitors

Compound 1 (Fig. 4A) is a reversible active-site directed inhibitor of SPL, featuring IC_{50} = 214 nM in a biochemical assay using purified human S1P lyase (manuscript in preparation). Using the assay for intracellular S1P described before [5], the compound increases S1P in HEK cells by a factor of 10 at a concentration of 14 nM.

In the current assay, compound 1 induced a concentration-dependent increase of S1P secreted by HEK293T cells, as indicated by the calcium flux assay on CHO-K1/$S1P_3$ cells (Fig. 4B); the EC_{50} value determined in four independent assays was 52 ± 21 nM. Without addition of Sph to the cell culture, the lyase inhibitor did not induce secretion of detectable S1P levels into the medium. As discussed previously [5], addition of Sph to cells appears to generate an intracellular pool of S1P that is susceptible to degradation by SPL; thus both intracellular [5] and extracellular S1P increase in response to an SPL inhibitor only, when cells are spiked with Sph. For the routine procedure, 1 μM Sph was included in the incubations, diluted in medium containing 4% charcoal-stripped FCS to minimize the adherence of the lipid to the plasticware. Optimal signals were obtained when the incubation time after addition of Sph was extended to 2.5 h; further prolongation did not increase S1P levels, while reduction to 1 h gave substantially lower levels.

The conditions for routine testing of SPL inhibitors are given under Materials and Methods. Following this protocol, we tested the SPL inhibitor 2 (IC_{50} for inhibition of purified enzyme: 2.4 μM) and its enantiomer 3 which is inactive in the biochemical assay (Fig. 4A). In agreement with the enzymatic data, 2 was found to be active (EC_{50} = 234 ± 102 nM, n = 4) whereas 3 was inactive (Fig. 4C), indicating that the secretion of S1P is indeed due to SPL inhibition.

The assay system described here is suitable for ranking of the cellular potency of active-site directed inhibitors of SPL which induce both intracellular S1P increase and S1P secretion. By contrast, deoxypyridoxine, 2-acetyl-4(5)-(1(R),2(S), 3(R),4-tetrahydroxybutyl)-imidazole, and LX2931 [4] fail to induce significant S1P increase within cells in culture [5], while clearly inhibiting SPL activity in animals. This is likely due to an indirect mode of SPL inhibition that becomes apparent only *in vivo*. As expected, these

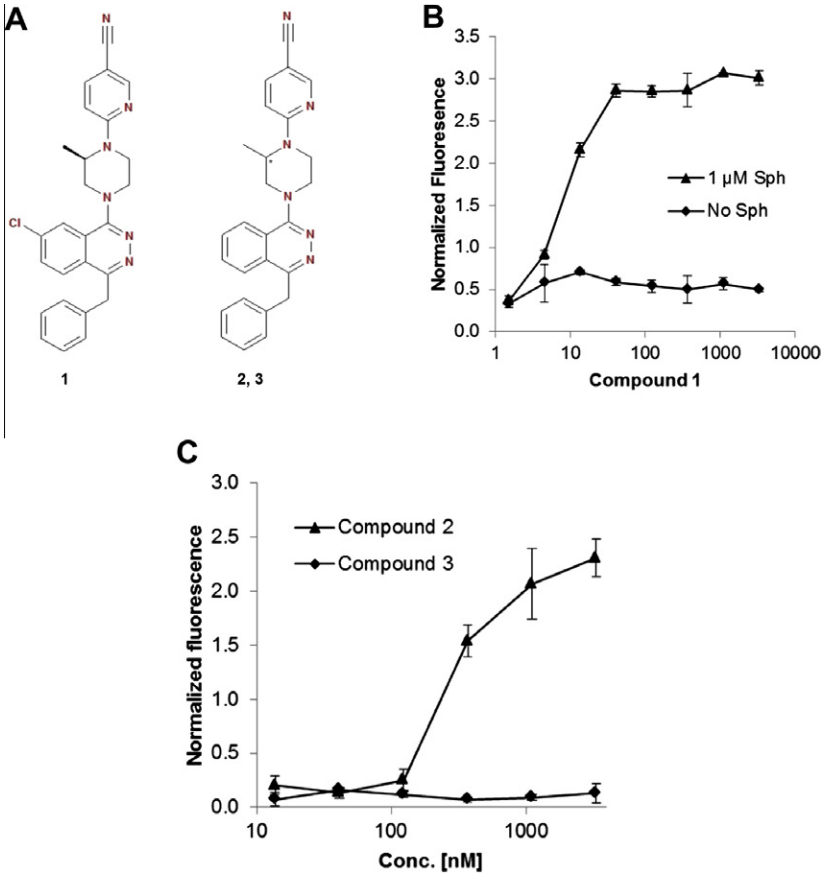

Fig. 4. Secretion of S1P from HEK293T induced by SPL inhibitors. Cells were incubated with graded concentrations of inhibitors as described under Materials and methods. Subsequently, the supernatants from the cell cultures were analyzed in the calcium flux assay. (A) Chemical structure of SPL inhibitors 1–3; compound 2 has R-configuration at the carbon marked with ∗, while the inactive enantiomer 3 has S-configuration. (B) Compound 1, tested in absence or presence of 1 µM Sph; (C) compound 2 and 3 in presence of 1 µM Sph.

compounds also did not induce detectable S1P secretion from HEK293T cells up to the highest test concentration of 100 µM.

In summary, we here provide a specific bioassay for the determination of S1P in culture supernatants; this assay can be used to determine the secretion of S1P from cells treated with SPL inhibitors, in order to demonstrate their ability to inhibit the enzyme within living cells.

We have successfully used this assay as a ranking tool during optimization of several classes of SPL inhibitors to enable the selection of suitable potent cell-permeable compounds for *in vivo* testing in experimental animal models.

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