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A practical process for the preparation of [^{32}P]S1P and binding assay for S1P receptor ligands

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HIGHLIGHTS

- Streamlined [^{32}P]S1P production process with reproducible radiochemical yield.
- Simplified assay of binding affinity for S1P receptors using [^{32}P]S1P.
- Reliable and repeatable IC_{50} values can be obtained by the reported method.

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ABSTRACT

Sphingosine-1-phosphate receptors (S1PRs) are important regulators of vascular permeability, inflammation, angiogenesis and vascular maturation. Identifying a specific S1PR PET radioligand is imperative, but it is hindered by the complexity and variability of current for binding affinity measurement procedures. Herein, we report a streamlined protocol for radiosynthesis of [^{32}P]S1P with good radiochemical yield (36–50%) and high radiochemical purity (> 99%). We also report a reproducible procedure for determining the binding affinity for compounds targeting S1PRs *in vitro*.

1. Introduction

Sphingosine 1-phosphate (S1P) is a potent bioactive lipid that regulates diverse physiological and immunological processes. Most of its best characterized actions are mediated through a family of five G-protein coupled receptors ($S1PR_{1-5}$) (Rosen et al., 2014). Since the development and approval of FTY720 (Gilenya®, Fingolimod) for oral treatment of relapsing-remitting multiple sclerosis (MS), the S1PRs have attracted a great deal of interest (Urbano et al., 2013). Diversity in the expression pattern and the function of S1PRs has been observed in the central nervous system (CNS). S1PR1, S1PR2 and S1PR3 are widely expressed in neurons and glia (no S1PR2 expression in astrocytes and oligodendrocytes), whereas expression of S1PR5 is highly restricted to oligodendrocytes (Soliven et al., 2011). S1PR1 and S1PR3 expression on reactive astrocytes significantly increased in both active and chronic inactive MS lesions (Van Doorn et al., 2010). Consistently, the functional antagonism for S1PRs is partly attributed to the direct neuroprotective effect of FTY720 (O'Sullivan and Dev, 2013).

The S1P/S1PRs pathway also plays an important role in both normal and pathological conditions outside the CNS system. S1PR1 and S1PR3 modulate angiogenesis by enhancing platelets, endothelial cell and vascular smooth muscle cell proliferation and migration, whereas S1PR2 receptors block the cell migration in the cardiovascular system (Swan et al., 2010). FTY720 and other S1PR1/S1PR3 antagonists showed potential atheroprotective effects in animal models of atherosclerosis and neointimal hyperplasia (Keul et al., 2007; Shimizu et al., 2012). Moreover, S1PR expression levels are associated with tumor growth, cancer staging, as well as patient survival (Kunkel et al., 2013; Yoshida et al., 2010).

Considering the importance of S1PRs in MS and other diseases, development of positron emission tomography (PET) radiotracers targeting specific S1P receptors would provide unique tools to detect the *in vivo* level of the corresponding receptor, and to monitor the physiopathological progression of these diseases (Inglese and Petracca, 2013; Kiferle et al., 2011; Kipp and Amor,

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2012; Miller et al., 2012). Until recently, there have been two radioligands developed that focus on the S1P receptors: BZM055 (Briard et al., 2011), an analog of FTY-720 that contains an ^{123}I on the aromatic core; and a recently published fluorinated analog of W146 (Prasad et al., 2014; Sanna et al., 2006). However, the lower brain penetrating capability of [^{123}I]BZM055 and the fast metabolism and defluorination *in vivo* of [^{18}F]W146 limited their clinical utility.

One of the hindrances for the development of PET agents for imaging S1PRs was a lack of a readily available quantitative binding assay for determining the binding affinity for compounds toward the S1P receptors. The functional assay, based on the downstream signaling responses following the receptor activation, is most commonly used. In the functional assay, the half maximal effective concentration (EC_{50}) represents the binding affinity of compounds, which actually reflects the ability to induce biological effects following the ligand-receptor binding and obviously only works for agonists (Gardner and Strange, 1998). In fact, the most efficient method of screening the binding affinity of novel compounds is with a competitive binding assay, using a ligand that is specific to the desired target protein(s); in this case radiolabeled S1P. While several fluorescently-labeled analogs of S1P have been prepared, none of these have been successfully applied to a binding assay targeting the S1P receptors (Ettmayer et al., 2004; Hakogi et al., 2003; Yamamoto et al., 2008). The radioactive competitive binding assay utilizing radiolabeled S1P provides a reliable measure of the binding affinity; and works equally well for agonists & antagonists (Gardner and Strange, 1998). Notably, a discontinuity between the more common functional assay results and the radioactive competitive binding assay results has been reported (Gardner and Strange, 1998; Hale et al., 2004). In order to carry out our ligand development project, we need to explore a binding assay method that could be performed in our laboratory routinely. We also needed to develop a detailed, reproducible procedure for the preparation of the required radioligand used in the binding assay. Herein, we report a simplified method to reliably produce ^{32}P-radiolabeled S1P, as well as a straightforward method for the competition binding assay.

2. Materials and methods

All reactions were performed in low-retention centrifuge tubes. Unless otherwise stated, all reagents were used as received. [γ-^{32}P]Adenosine triphosphate (ATP) (370 MBq/mL; specific activity = 222 TBq/mmol) was purchased from PerkinElmer (Boston, MA). Recombinant human sphingosine kinase 1 (rhSPHK1) was purchased from R&D Systems (Minneapolis, MN), upon receipt, it was aliquoted into ten parts diluted with buffer A (*vida infra*) to a final concentration of 1 μg/10 μL. $S1PR_1$ membranes that were prepared from Chinese hamster ovary (CHO)-K1 cells expressing recombinant human $S1PR_1$ receptors and were used in the study were purchased from Chan Test Corp. (Cleveland, OH). $S1PR_{2\text{-}3}$ membranes that were prepared from Chem-1, an adherent cell line expressing the promiscuous G-protein, Gα15, were purchased from Merck Millipore (Billerica, MA). All the membrane preparations are crude membrane preparations made from proprietary stable recombinant cell lines to ensure high-level of $S1PR_{1\text{-}3}$ surface expression. The kinase aliquots and membranes were stored at −80 °C until use.

2.1. Establishment of the calibration curve

Known volumes (1.48–3.7 MBq) of commercially available [γ-^{32}P]ATP were added to scintillation vials and the radioactivity (counts per minute, CPM) was measured by a Beckman LS 3801 scintillation counter using Cherenkov counting. The readout and the calculated radioactivity were plotted to establish the calibration curve (through the origin) and the equation.

2.2. Radiosynthesis of [^{32}P]sphingosine 1-phosphate

[^{32}P]S1P was prepared by addition of 12 μL 1 M $MgCl_2$ and 65 μL 1 mM sphingosine in 5% Triton X-100 to a 2 mL low-retention centrifuge tube. 1.0 μg sphingosine kinase 1 in 10 μL Buffer A (100 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) [pH 7.5], 10 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol, protease inhibitor cocktail) was then added to the reaction mixture. The volume was adjusted to 1 mL with sphingosine kinase buffer (20 mM tris(hydroxymethyl)aminomethane (Tris) [pH 7.4], 1 mM EDTA, 0.5 mM 4-deoxypyridoxine, 1 mM β-mercaptoethanol, 1 mM orthovanadate, 40 mM β-glycerophosphate, 10% glycerol, and protease inhibitor cocktail). 8.51 MBq [γ-^{32}P]ATP was then added to the reaction vial, which was mixed by pipette. The reaction was then incubated in a 33–35 °C water bath for 2 h, at which time the reaction mixture was divided into two 2 mL centrifuge tubes. To each of these tubes was added 0.5 mL $CHCl_3$, 0.5 mL MeOH, and 50 μL 3 M $NaOH_{(aq)}$. The centrifuge tubes were then vortexed for ~30 s and centrifuged for 10 min@1500 g to separate the phases. The upper aqueous layer were transferred to 2 mL low-retention centrifuge tubes, 0.5 mL $CHCl_3$ and 100 μL 12 M HCl were added to each tube. The tubes were vortexed for ~30 s and then centrifuged for 10 min@1500 g to separate the phases. The upper aqueous layers were removed and discarded. The lower organic layers were combined and reduced under a stream of $N_{2(g)}$ to give the [^{32}P]S1P. The [^{32}P]S1P was then dissolved in 500 μL of dimethylsulfoxide and transferred to a low-retention centrifuge tube for storage. Aliquots of 1, 2, and 3 μL were removed and the radioactive yield was determined by Cerenkov counting (Beckman LS 3801 scintillation counter) to be 3.7 MBq using the calibration curve described above. The radioactive purity was determined by radio TLC eluting with an *n*-butanol/acetic acid/water (3/1/1, v/v/v) solvent system. The desired [^{32}P]S1P has an R_f of 0.5, and the radio-purity was determined to be 95%. This R_f matches the known literature value for this solvent system (Maceyka et al., 2007), as well as the value for cold S1P (visualized by potassium permanganate staining).

2.3. Binding assay

The binding affinity of S1P ligands was measured by [^{32}P]S1P competitive ligand binding assay. The assay buffer consists of 50 mM HEPES–Na (pH 7.5), 5 mM $MgCl_2$, and 1 mM $CaCl_2$, 0.5% fatty acid-free bovine serum albumin (BSA). $S1PR_{1\text{-}3}$ membranes were diluted in the assay buffer to yield 1–2 μg/well, a final concentration for 96-well plates, and kept on ice. Test compounds were dissolved in dimethylsulfoxide (DMSO) or methanol at a high concentration (1–10 mM). The solutions were then diluted in the assay buffer to yield various final concentrations (100 μM to 0.001 nM). [^{32}P]S1P working solutions were diluted in the assay buffer to obtain a final concentration of 0.1–0.2 nM.

Test compounds in the assay buffer (50 μL) were pre-incubated with $S1PR_{1\text{-}3}$ membranes (50 μL) for 30 minutes at room temperature. [^{32}P]S1P working solutions were added to give a final volume of 150 μL, 0.1–0.2 nM [^{32}P]S1P and 1–2 μg membrane protein per well. binding was performed for 60 min at room temperature and terminated by collecting the membranes onto 96-well glass fiber (GF/B) filtration plates (Millipore, Billerica, MA), which had been presoaked with 100 μL assay buffer per well for 60 min. each filter was washed using 200 μL of the assay buffer five times. the filter bound radioactivity was measured by a Beckman LS 3801 scintillation counter using Cherenkov counting.