SPHINGOLIPIDS AS TARGETS FOR RECOVERY FROM SPINAL CORD INJURIES: DECIPHERING THE ROLE OF SPHINGOSINE-1-PHOSPHATE

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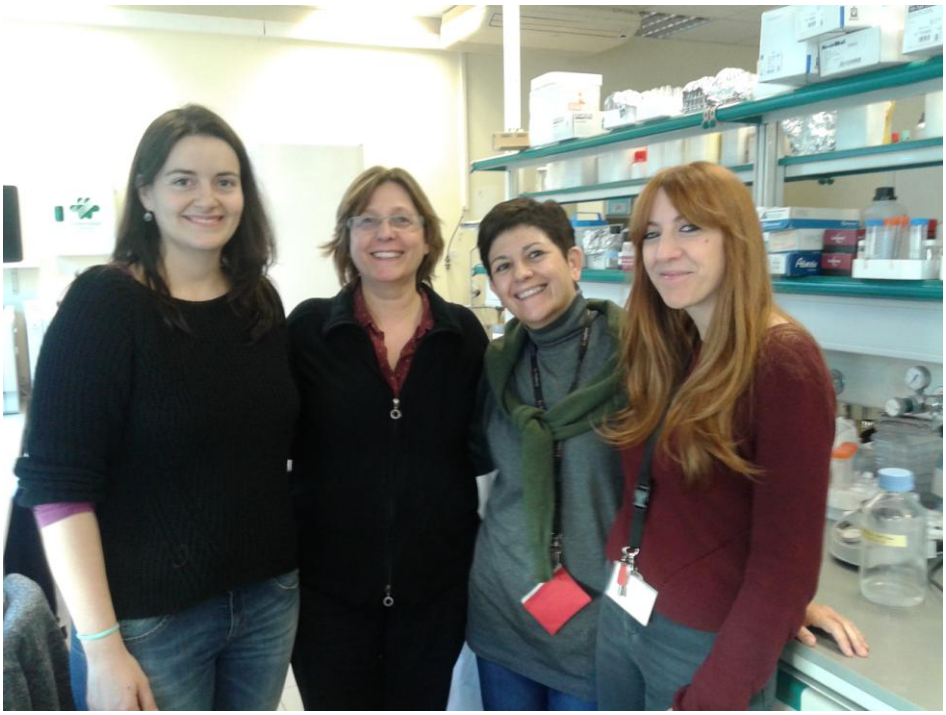


1. Summary

Accumulated evidence indicates that D-erythro-sphingosine-1-phosphate (S1P) modulates cellular processes triggered after cell injury (cell death, cell apoptosis, lipid hydrolysis, oxidative stress, tissue damage) and promotes cell growth, trophic activity, angiogenesis and neurogenesis. Thus, the discovery of new neuroprotective therapies for spinal cord injury (SCI) can arise from interventions on S1P metabolism aimed at increasing S1P levels. This can be achieved by inhibiting S1P lyase (SPL), the enzyme responsible for the irreversible S1P degradation. Therefore, SPL could emerge as a novel neurorestorative target.

Based on X-ray structures reported for SPL, we have designed and synthesized three generations of inhibitors, which have been tested using a high throughput screening method set up in the frame of this project. The third generation compounds, suitably vehiculized, could provoke an increase in S1P at the site of the SCI. Moreover, we have found that the S1P concentration is modulated by the amounts and activity of SPL present in the spinal cord injured area, while other S1P metabolizing enzymes such as kinases and phosphatases are not involved. Consequently, modulating SPL activity seems to be a good strategy for controlling the concentration of S1P in lesioned spinal cord.

2. Results

The general objective of this proposal was to demonstrate that chemical modulation of SPL activity could provide the basis for future pharmacological interventions to improve functionality of a damaged nervous system.

First of all, we have shown that after experimental spinal cord (SC) injury, alterations in the SC sphingolipids occur mainly for sphingoid bases and, especially S1P. The most important change occurs at day 3 post-lesion, when the S1P/So ratio decreases as compared to other time points (Figure 1).

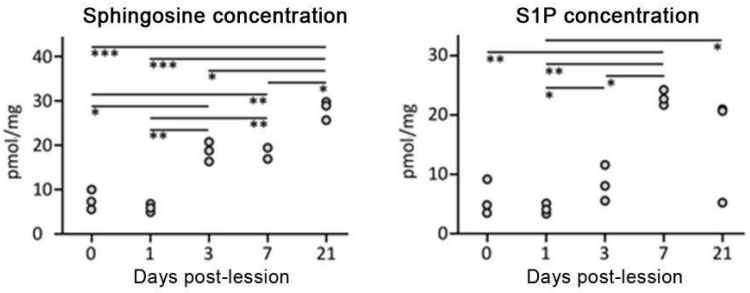


Figure 1. Sphingosine and S1P concentrations in the spinal cord of mice at different times after the lesion, as determined by liquid chromatography-coupled to mass spectrometry.

In order to understand the mechanisms involved in the changes in the SC So/S1P ratio at day 3 post-lesion, the levels of enzymes involved in S1P metabolism were measured. Western blot analysis showed that SPL exhibited the main alteration (Figure 2A). The enzyme levels increased at day 1 to decrease time-dependently up to day 7. Transcript levels were also determined for SPL. As shown in Figure 2B, SPL mRNA decreased after the damage over the time post-injury, in disagreement with the observed changes in protein levels. These results suggest that increases in protein are probably due to reduced degradation. Other enzymes of S1P metabolism were not significantly affected (data not shown).

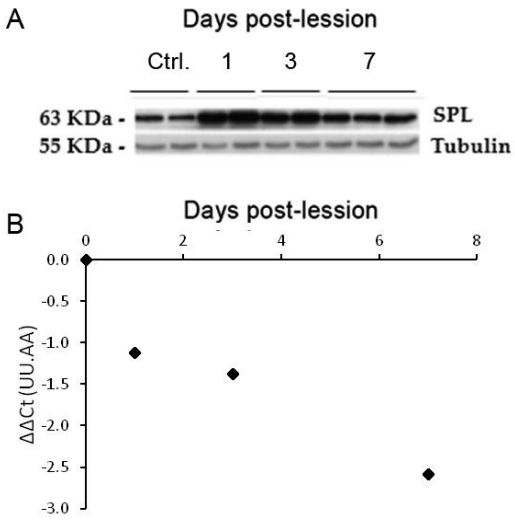


Figure 2. SC SPL levels as determined by Western blot (A) and mRNA SPL levels as measured by qPCR at different times after the SC injury. Data were obtained from 2-3 replicates.

In order to further assess the putative role of the S1P/SPL axis in experimental SC injury, SPL +/- transgenic mice were used. These animals recovered the motor function after the injury, whereas the wild types did not (Figure 3).

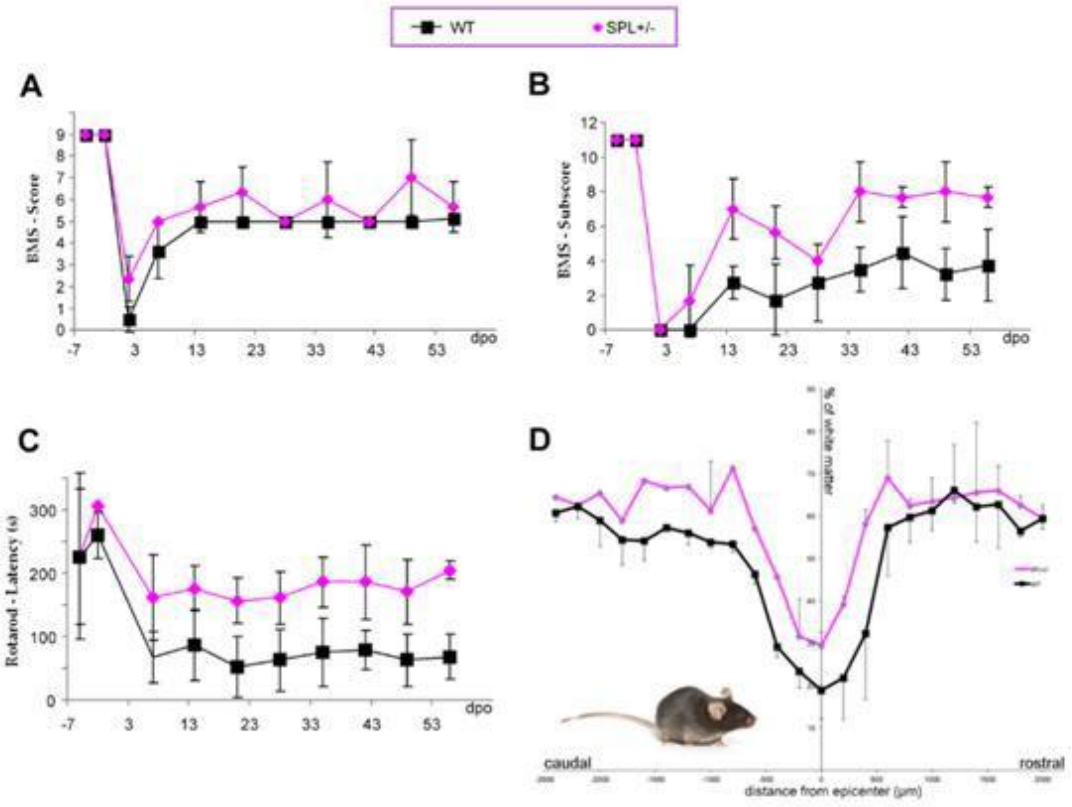


Figure 3. Motor function recovery after experimental SC lesion.

In another approach, the role of S1P/SPL in the recovery after the experimental SC lesion was investigated using chemical inhibitors designed and synthesized as described in the following paragraphs.

- *Homology modeling from bacterial SPL and design of a virtual library of potential inhibitors***.**

A homology model was built using templates from yeast SPL and *Symbiobacterium thermophilum* SPL. This model was used to build a series of potential inhibitors, which were filtered by virtual screening techniques to afford a set of 12 compounds from condensation of a series of carboxylic acids with a common aniline scaffold (see Figure 4).

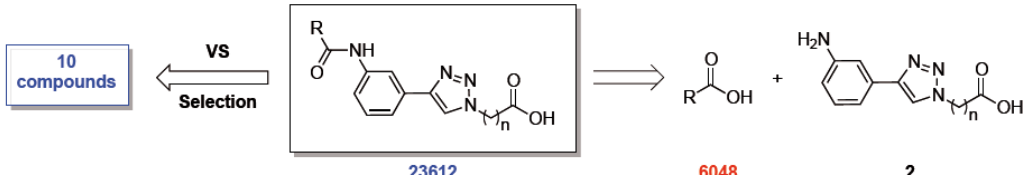
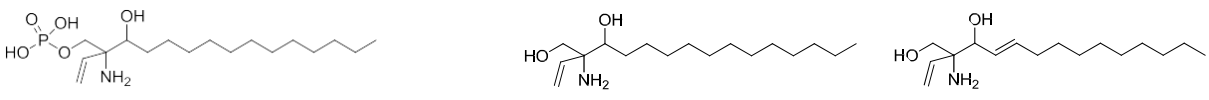


Figure 4. Compounds derived from a common aniline scaffold resulting from virtual screening

Unfortunately, the inhibitory activity of the resulting compounds was modest. This may be due either to an overestimation of the electrostatic interactions predicted by the docking protocols or to the inability of the compounds to reach the enzyme active site.

- *Synthesis of 2-vinylsphingosine-1-phosphate (2VS1P) as potential mechanism-based inhibitors*

Compound **RBM6-24** (as mixture of isomers, see Figure 5) was obtained from a multistep reaction sequence. A mixture of the 4 isomers and separate mixtures of the corresponding *syn* and *anti* diastereoisomers was also obtained and submitted to enzyme activity assays. Similarly, aminodiols **RBM6-31** and **JG-1** were also obtained as mixtures of four and two isomers respectively.



RBM6-24 (mixture of isomers)
RBM6-24-A *(rac)anti;* **RBM6-24-B** *(rac)syn*

RBM6-31

JG-1 *(rac)anti*

Figure 5. Potential mechanism-based SPL inhibitors obtained by total synthesis

- *Non-reactive enzyme intermediate and substrate analogs as potential SPL inhibitors*

RBM7-12 and **RBM7-32** were designed as non-reactive analogs of the putative intermediate of the degradation of dihydrosphingosine (dhSph) and sphingosine (Sph), respectively, by SPL (Figure 6).

Inhibitory activity of **RBM6-24** and non-reactive enzyme intermediate analogs (**RBM7-12** and **RBM7-32**) was tested against hSPL and N2 neural cell line in culture (Table 1).

Table 1. Effect of compounds on SPL activity.

	IC_{50} (μM)	
Compound	**hSPL**	**N2cells [a]**
RBM6-24	12.1	42.1
RBM6-31	inactive	inactive
JG-1	inactive	36.3
RBM6-24A	6.4	6.0
RBM6-24B	89.2	23.9
RBM7-12	81.1	19.4
RBM7-32	89.0	23.9

[a]Intact N2 cells were exposed to different concentrations of the compounds for 24 h and SPL activity was determined in cell lysates using a fluorogenic assay as reported (Bedia et al. Chembiochem. 2009, 10, 820).

The most potent and non toxic compounds (RBM6-24A and RBM7-32) were then evaluated for their ability to elicit cytoprotection. Unfortunately, the compounds did not provoke an increase in cell survival upon exposure to apoptotic stimuli.

In order to evaluate SPL inhibition by RBM6-24A and RBM7-32 in vivo, the compounds were administered via i.p. to mice. However, no differences in the SC SPL activity were observed between control and treated animals. To rule out the possibility of a defective blood-brain barrier crossing, inhibition was also tested ex vivo (in tissue homogenates) but inhibition was not observed either. These results indicated that higher concentrations of inhibitors or more potent compounds are required to block SPL activity in animals.

Subsequently, a series of stereodefined azidosphingosine phosphates was designed as substrate analog inhibitors by replacement of the substrate amino group with non-reactive azide surrogate (Figure 6). The series result from the formal replacement of the amino group in S1P and dhS1P with a non-reactive azido moiety. Based on mechanistic considerations, the amino group in S1P is crucial for the reaction with PLP as the first step of the catalytic process by which SPL splits S1P into 2-hexadecenal and 2-aminoethanol phosphate. From a structural standpoint, the azido group is able to occupy the amine pocket in the enzyme active site. In order to explore the stereoselectivity of the enzyme towards our azido analogs, the eight possible stereoisomers at C2 and C3 positions were synthesized, as indicated in Figure 6.

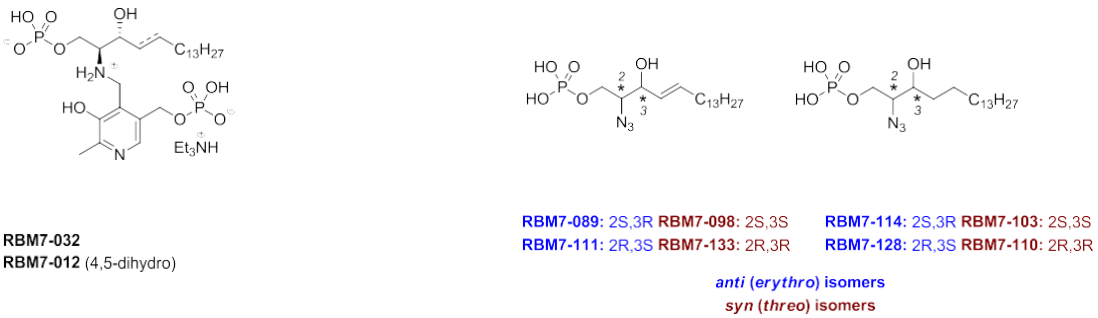


Figure 6. Non-reactive SPL reaction intermediates and substrates

As shown in Table 2, the new compounds exhibited inhibitory activity against hSPL in the low micromolar range, using RBM13 at 125 μM as substrate.

Table 2. Effect of compounds on SPL activity.

Compound	IC50 (μM)
RBM7-89	10.1± 1.6
RBM7-98	28.8±4.0
RBM7-111	5.2± 0.8
RBM7-133	22.9±4.9
RBM7-114	25.7±3.4
RBM7-103	21.8±0.4
RBM7-128	10.8±0.3
RBM7-110	28.3±0.4

On the other hand, docking studies against human SPL confirmed that all azido-phosphate stereoisomers are active against human and bacterial SPL and also that the stereochemistry of the azido alcohol fragment, as well as the presence/absence of a double bond at C4-C5 position, are not crucial for SPL activity. However, additional experiments confirmed that the presence of a phosphate group at C1 is essential for SPL activity

- *Synthesis of improved SPL probes for enzyme activity determination*

Compound RBM13 (Figure 7) is a fluorogenic probe that was developed in our group for the in vitro determination of SPL activity. Despite its remarkable selectivity, the moderate affinity for SPL shown by this probe in enzyme assays prompted us to look for structurally modified, "second generation" improved substrates. Based on structural considerations of the enzyme active site, we reasoned that the probe affinity could be enhanced by increasing the distance between the bulky coumarin fluorophore and the amino phosphate moiety present in RBM13. With these considerations in mind, we synthesized the elongated analogs RBM7-077 and RBM7-148 shown in Figure 7.

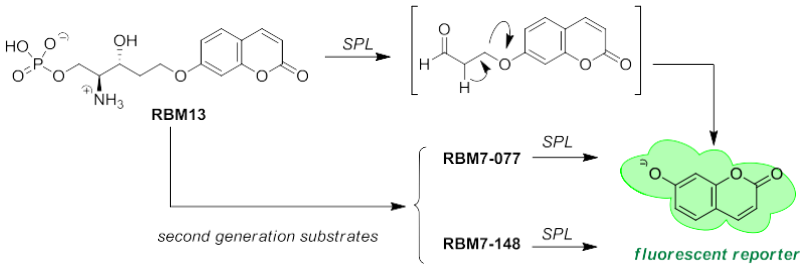


Figure 7. Original SPL substrate RBM13 and "second generation" analogs RBM7-077 and RBM7-148

As shown in Table 3, the new probes turned out to be sensibly better SPL substrates than the original one. Moreover, these probes, which are not cell-permeable, can be incorporated in nanoparticles and delivered to the cells.

Table 3. Kinetic parameters of hSPL

Substrate	K_M (mM)	V_{max} (pmol·min^{-}	V_{max}/K_M
RBM13	2071.7 ± 143.2	110.0 ± 23.8	0.05
RBM7-077	132.2 ± 14.2	262.7 ± 13.1	1.99
RBM7-148	509.4 ± 80.5	441.6 ± 21.9	0.87

3. Relevance and possible implications

1. *Lipid profile (sphingolipids) as biomarkers of spinal cord injury*. The analyses performed in both murine model and human plasma samples indicate the occurrence of changes in some species, besides S1P. These changes suggest new therapeutic targets. We should expand the number of samples analyzed to validate the preliminary results.

2. *Modulation of intracellular and extracellular concentration of S1P through actions on SPL in the central nervous system is a viable strategy in the pathophysiology of spinal cord injury*. The results of the characterization of the sphingosine/S1P/S1PR pathway after spinal cord injury indicate that the concentration of S1P depends exclusively on the concentration of sphingosine at short times (up to 24 hours) and it is then modulated by the concentration and activity SPL.

3. *Use of HTS methods to determine SPL activities.* The novel methods make it possible to perform enzyme activity studies in both cells and tissues quickly and simply.

4. *Ability to modulate S1P concentration by SPL inhibitors*. Proper administration of the third generation of compounds obtained in the frame of the project could cause significant increases in S1P in the site of injury.

4. Publications

Cingolani F, Casasampere M, Sanllehi P, Casas J, Bujons, J, Fabriàs G. Inhibition of dihydroceramide desaturase activity by the sphingosine kinase inhibitor SKI II. *J Lip Res*. **2014**.55(8):1711-1720.

Sanllehí P, Abad JL, Casas J, Delgado, A. Inhibitors of sphingosine-1-phosphate metabolism (sphingosine kinases and sphingosine-1-phosphate lyase) *Chemistry and Physics of Lipids* **2016,** 197:69-81

Casasampere M, Ordoñez YF, Pou A, Casas J. Inhibitors of dihydroceramide desaturase 1: therapeutic agents and pharmacological tools to decipher the role of dihydroceramides in cell biology. *Chem Phys Lipids*. **2016**. 197:33-44

Del Aguila A. *et al*. Alterations in the sphingosine-1-phosphate signalling system following Spinal Cord Injury. In preparation with members of subproject 2.

Sanllehi, P et al. Design and synthesis of new coumarin-based sphingosine-1-phosphate lyase (SPL) probes. In preparation.

Sanllehi, P, et al. New mechanism-based sphingosine-1-phosphate lyase (SPL) inhibitors. In preparation.

Calderón, R et al. Irreversibility of sphingosine 1-phosphate lyase inhibition by 2 vinyldihydrosphingosine 1-phosphate stereoisomers. In preparation.