Design, synthesis and biochemical characterization of Fingolimod analogs for targeting PP2A

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Aim of the project
Fingolimod (also known as FTY720) is a recently discovered drug which is used for the treatment of Multiple Sclerosis (MS). MS is the most common chronic inflammatory disease of the central nervous system and is the leading cause of disability in young adults in the Western hemisphere (1). The drug’s action consists in interfering with sphingolipidic metabolism. Sphingomyelin in the plasmatic membrane is the substrate of sphingomyelinase enzyme, which gives ceramide; ceramidase deacylates ceramide into sphingosine. After that, the enzyme Sphingosine Kinase (SK) leads to the generation of Sphingosine-1-phosphate (S1P). S1P belongs to the lysophospholipid group and mediates many cellular processes, such as cell proliferation and survival, cell motility, tissue invasion, angiogenesis and the migration of immune cells (1). The balance between the concentration of ceramide and S1P in the cell can also influence its survival through a rheostat mechanism: while S1P enhances the survival of several cell types, its precursors (sphingosine and especially ceramide) promote the arrest of the cell growth and cell death (2). Another important element is that ceramide activates the enzyme PP2A, a Ser/Thr phosphatase which acts on SK by dephosphorilating – and so inactivating – it on Ser 225 residue (3).

Several external factors, such as growth factors and cytokines, have the direct consequence of increasing the level of S1P through the activation of SK1, an isoform of SK. Indeed, two types of SK exist in men: SK1 (subtypes a and b), over-expressed in several cancer types because of its anti-apoptotic action, and SK2, apoptotic. For being active, both the isoforms have to be phosphorilated by the ERK1 or ERK2 enzymes on the Ser225 residue. After the linking to an agonist, SK can either be translocated in the plasmatic membrane or regulate nuclear transcription, or even both, but all these mechanisms lead to the same effect, i.e. the increasing of the levels of S1P (4).

The newly made S1P can act in two ways, included in the so-called “inside-out signaling”: it can either be secreted outside the cell and act in an autocrine or paracrine way, stimulating the membrane receptors for S1P itself (called S1PRs) or it can act inside the cell as a second messenger (2). The extracelullar action of S1P is linked to the presence of five specific G-coupled protein receptors (S1PRs), which are present in all the cell types (but S1PR4, only in lymphocytes, and S1PR5, only in olygodendrocytes and NK cells) and which can lead to several effects, such as cell proliferation, anti-apoptotic phenomena (this means also tumor cells growth), mutations in the cytoskeleton, cell-cell interaction and, above all, the migration of the lymphocytes from the secondary lymphoid organs. The last one is the effect which is involved in Fingolimod action.

Fingolimod is a prodrug commercialized by Novartis that comes from myriocin, a metabolite of Isaria sinclairii and Myrothecium verrucaria fungus. When administered, it is activated by phosphorilation thanks to SK2; this leads to a S1P-mimetic that can link the S1PR1/3/4/5 receptors with high affinity (the fact that FTY720 doesn’t work on S1PR2 has a positive meaning for the drug function itself: indeed, this is the receptor which is involved in the down regulation of the migration of macrophages and their recruitment to the inflammation sites). In the beginning the drug-receptor bond activates the signaling pathways that would be the consequence of the normal interaction between S1P and S1PRs (agonism). However, after prolonged administration the action of FTY720 is no more an agonist, but a functional antagonist one: the
overstimulation of S1PRs leads to their down regulation (5). For being more specific, this drug acts especially on S1PR1, which is internalized and consequently degraded; this leads to a decrease in the gradient of S1P between the tissues and the blood vessels, and this is particularly relevant for the lymphocytes: this reduces the responsiveness of T cells to the egress signal S1P and favours CCR7-mediated retention in lymph nodes, as shown in Figure 1 (6).

![Figure 1. Model of T cell retention by Fingolimod in lymph nodes (6).](image)

This phenomenon is extremely relevant in MS, because this pathology gives auto-immune attack of nerves mediated by T lymphocytes; if they cannot leave the lymphoid organs, the damage to the nerves is slowed down. However, it has been demonstrated that this doesn’t lead to lymphopenia. The second relevant action mediated by this drug is the activation of PP2A without even being phosphorilated (3). The aim of this project is to find out structural Fingolimod analogs with high affinity and selectivity for PP2A: indeed, this enzyme is involved in many pathologies besides MS, e.g. some forms of tumors. It is well known, indeed, that PP2A acts as a tumor suppressor and plays a crucial role in the regulation of cell cycle progression, survival and differentiation. It has been also shown that functional loss of PP2A activity is important for some myeloid malignancies such as acute myeloid leukemia (AML), as demonstrated by the positive results of the treatment of AML cells with FTY720, and also chronic myelogenous leukemia and BCR/ABL-driven leukemias (7,8).

**Methods**
The project will proceed cyclically through different stages:

1. the design of new Fingolimod analogs or new compounds with the same target. The design of new compounds will consider the state of the art and the result of our, in house, experience. By now we decided to start from precursors as eugenol (a natural derivative that comes from clove oil) or allyl benzene that are easily available.

2. synthesis of the compounds planned in step 1. The laboratore where I am never worked on similar compounds before; this means that we should set up new synthetic pathways. For this a robust literature search will help.

3. elaboration of an highly performing strategy of purification: since the precursors are in an oily state, this will probably mean preparative flash chromatography or HPLC. The characterization will include the use of several techniques such as analytical HPLC, HRMS and NMR (both 1D and 2D).
4. biological tests on Fingolimod analogs, which will be performed following a three steps schedule: first of all, they will be tested on the isolated PP2A enzyme. After that, if the results will be encouraging, they will be tested on isolated cells; in particular, the privileged cell line will be the murine HSC (Hepatic Stellate Cells) one. HSCs are liver cells that, thanks to their lipid droplets, contain the biggest part of the endogenous retinol. The third step will be in vivo tests on mice, to find out the effects of the compounds on living systems.

5. evaluation of the biological tests results to elaborate SAR hypothesis on the compounds; this can lead again to step 1: design of new compounds that could best fit the aim of the project.

Timeline for the project
The project will progress ciclically following the steps depicted above. The duration of each step can vary a lot from step to step.

References:

2) Zanin, M., Identificazione dei recettori specifici per S1P nel muscolo soleo di ratto. Ruolo dei derivati della sfingomielina sul trofismo del muscolo scheletrico. Tesi di dottorato in Scienze Mediche, Cliniche e Sperimentali, indirizzo Neuroscienze, Università degli studi di Padova, ciclo XX.


