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Are We Using the Right Pharmacological Tools to Target EphA4?

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ABSTRACT: The EphA4 receptor has been proposed to be a key actor in neurodegenerative diseases. In the last years, several research groups focused their efforts on the discovery of small molecules capable of blocking EphA4 activity by binding its extracellular domain. However, none of the compounds so far identified possess adequate chemical and/or pharmacological profiles to assess the "druggability" of EphA4 in animal models. New efforts are required to deliver a new generation of suitable pharmacological tools.

KEYWORDS: *Eph-ephrin antagonist, EphA4, compound* **1**, *Rhynchophylline, UniPR129*

The Eph−ephrin system is emerging as a new target for the treatment of several diseases including cancer, diabetes, and inflammation. Currently, the Eph−ephrin system is becoming more and more attractive to neuroscientists considering the key role played by EphA4 receptor in both Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS).

The prominence of EphA4 receptor in AD and in ALS has been revealed through the application of well-established experimental approaches including gene-based techniques and protein-based assays, with the last making use of monoclonal antibodies, recombinant proteins, or peptides as effective pharmacological tools. While these tools allow demonstrating the existence of a clear connection between a disease and a molecular target, they do not give an exhaustive description of the "druggability" of the target. To answer this question, a "drug hunter" often needs small-molecules as pharmacological tools. For the Eph−ephrin system and specifically for the EphA4 receptor, "druggability" still remains an open issue, as available small molecules suffer for inadequate physicochemical properties (i.e., low chemical stability) and/or for poor pharmacodynamic (low potency or lack of specificity) and pharmacokinetic profiles, limiting or even preventing their application in *in vivo* studies. Let us make three straightforward examples about the inadequacy of available EphA4 antagonists

1. THE IMPORTANCE OF BEING PURE AND STABLE

4-(2,5-Dimethyl-1*H*-pyrrol-1-yl)-2-hydroxybenzoic acid (compound **1**, Figure 1), probably the most commonly used EphA4 antagonist, is a chemically unstable product which undergoes spontaneous transformation when exposed to air and light. The discoverers of **1** themselves, 3 years after its disclosure, stated that " *...the newly synthesized compound* **1** *did not show detectable inhibition of ephrin-A5 binding to EphA4-Fc in ELISAs or EphA2 phosphorylation in cells stimulated with ephrin-A1 Fc. ...when left exposed to air at room temperature in dry form, compound* **1** *acquired a progressively darker brown color. Concomitantly, the compound became progressively more active in ELISAs measuring inhibition of ephrin-A5-EphA4 binding.*"[1] Further studies clarified that compounds of the same chemical class of **1** spontaneously forms a mixture of reactive polymers, with a molecular weight of 40 kDa, which are likely responsible for the aspecific biological activity of 2,5-dimethyl-pyrrol-1-yl-benzoic acid class.[2]

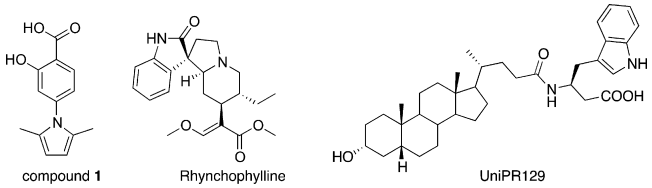

Figure 1. Small molecules claimed to target the Eph4 receptor here reviewed.

2. THE IMPORTANCE OF BEING ACTIVE

The *Uncaria rhynchophylla* alkaloid rhynchophylline has been recently shown to rescue impaired synaptic transmission in models of AD by blocking the ephrin-dependent activation of the EphA4 receptor.[3] Considering the plethora of pharmacological activities shown by rhynchophylline,[4] and in particular those related to neuroprotection, that is, (i) inhibition of calcium channel activity, (ii) modulation of potassium channel activity, and (iii) inhibition of NMDA receptors, it is remarkably difficult demonstrating that rhynchophylline improves neuronal plasticity by targeting the EphA4 receptor only. While we have no problems in believing that EphA4 receptor could be a target to look at for AD, specific concerns emerge analyzing the raw data reported by Fu et al.[3] regarding the effective ability of rhynchophilline to bind and inhibit EphA4. For instance, these authors showed (through a pull-down assay) that biotinylated rhynchophylline coprecipitates with EphA4-Fc, a chimeric protein composed by the Fc region of immunoglobulin and the extracellular domain of EphA4. However, the presence of detectable signals in the control, along with the lack of control experiments involving "cold" rhynchophylline or the EphA4 receptor alone do not allow to rule out if rhynchophilline binds the extracellular domain of the EphA4 receptor. The authors also claimed that rhynchophylline

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inhibited EphA4 phosphorylation in rat neurons. However, the poor reduction of EphA4 phosphorylation (20−25% of inhibition), with an unknown concentration of rhynchophylline, was not supported by a cell viability study or by an inhibition assay on the isolated kinase domain of EphA4. Finally, the absence of a measure of the potency (i.e., IC_{50} value) of rhynchophylline for any of these assays raises serious doubts about the real activity of this compound on EphA4. We thus tested rhynchophylline for its ability to interfere with EphA4-ephrin-A1 binding and to inhibit the EphA4 kinase activity using an ELISA-binding protocol developed in our lab or the LANCE technology. In both assays, rhynchophylline was shown to be inactive up to 50 μM.

3. THE IMPORTANCE OF BEING BIOAVAILABLE

Small molecules targeting the EphA4 receptor with a reasonable affinity and specificity have been also reported in literature by our group. Among these compounds, UniPR129[5] was identified as a low micromolar antagonist of the Eph receptor able to block cell-dependent signals transduced by the Eph−ephrin system without exerting cytotoxic effects. While promising as an *in vitro* tool, the usefulness of this compound is limited by its low oral bioavailability as indicated by pharmacokinetic data obtained in our lab.

■ CONCLUDING REMARKS

During the past decade, pharmacological tools able to inhibit Eph receptors have been reported in literature and they are currently being applied to investigate the role of the Eph−ephrin signaling system in the onset and progression of central nervous system diseases, including AD and ALS. However, none of them is suitable for *in vivo* studies due to stability (compound **1**), selectivity (rhynchophylline), activity (compound **1** and Rhyncophylline), or pharmacokinetic issues (UniPR129).[5] Much more work is required to discover and optimize small molecules suitable for validating EphA4 receptor as novel drug target for neurodegenerative diseases.

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Notes

The authors declare the following competing financial interest(s): The Authors are inventors in a patent application protecting novel Eph receptor antagonists.

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