DEVELOPMENT NEW LRRC8/VRAC CHLORIDE CHANNEL INHIBITORS, A NEW KEY ELEMENT IN ISCHAEMIA

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1. Abstract of the project

Ischemic insults such as stroke cause astrocytes to undergo sustained swelling, which activates the volume-regulated anion channel (VRAC). This activation causes glutamate release in extracellular spaces contributing to the observed dramatic increase in the concentrations of glutamate in stroke. Glutamate contributes to excitotoxic damage. Administration of VRAC inhibitors in animal models of stroke reduces the intraischemic glutamate release and the brain infarction area. However, the inhibitors used lack specificity and show poor penetration through the blood brain barrier (BBB). It has recently been identified that the VRAC channel is formed by heteromers of LRRC8 proteins. Thus, it is now possible to measure its function in a simple system as Xenopus oocytes using two-electrode voltage clamp or cell lines using flow cytometry.

In this project we propose to find novel specific and BBB-permeable LRRC8 inhibitors that could be used for therapy of stroke. We propose an integrated, multidisciplinary and iterative approach between a medical chemistry group which will synthesize novel derivatives of two known VRAC/LRRC8 inhibitors (DCPIB and carbenoxolone) and an electrophysiologist/cell biologist group that will test the novel compounds on two different cell systems. The two selected hits will be prepared and modified through conventional techniques looking for improved potency against VRAC and, importantly, selectivity. This will include the programmed structural modification of meaningful moieties in their respective molecules through well-established synthetic transformations. Also, in a further tuning, once the best inhibitors have been selected, these compounds will be optimized for activity in the central nervous system, especially aiming at an efficient crossing of the BBB. Here, a pro-drug approach will be pursued. Our aim is to find one/two compounds that will selectively inhibit the LRRC8/VRAC channel to test in a rodent model of stroke.

2. Results

Task 1. Detailed setup of the screening system. 1st Year (January- April)

Initially, two different systems were proposed to screen for inhibitors of the VRAC channel. HAP1 cells deficient for the LRRC8A protein (the main component of the VRAC channel) were resistant to the antibiotic blasticidin as it cannot enter the cells. Our first

proposal was to apply VRAC inhibitors and see whether they can protect wild-type cells from the cytotoxic effects of blasticidin. However, the inhibitor CBX was not able to protect wild-type HAP1 cells and even killed them. We reasoned that HAP1 KO cells without VRAC have been adapted to the lack of the channel, whereas an acute inhibition will kill the cells independently of blasticidin. Thus, we did not screen the inhibitors using this assay and we concentrated only on measuring the activity of the channel using electrophysiology. Thus, we mainly used Xenopus oocytes expressing GFP tagged LRRC8 proteins, as initially planned. In addition, we performed some whole-cell patch clamp measurements in HEK293 LRRC8 knockout cells expressing LRRC8 proteins after constructing different expression plasmids. Finally, we also performed some whole-cell patch clamp measurements in primary cultures of astrocytes expressing endogenous proteins, to prove that the inhibitors were able to inhibit VRAC current in astrocytes. Thus, we concluded that the screening system had been setup correctly.

Task 2. Screening of the inhibitors provided by the MedChem group
CBX derivatives

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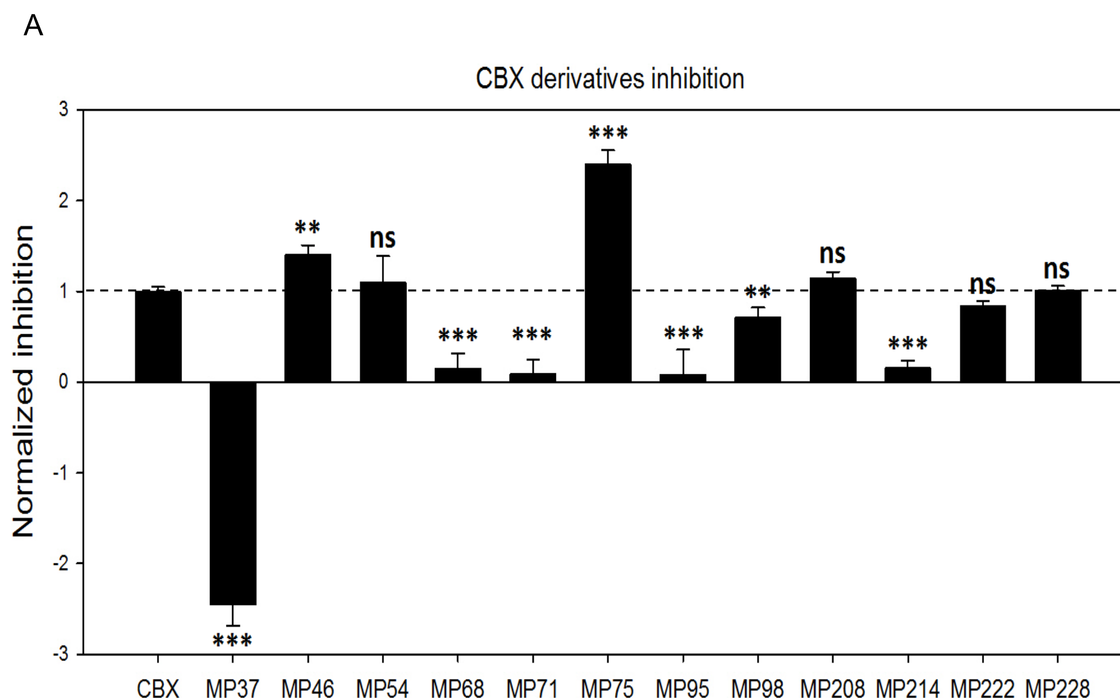

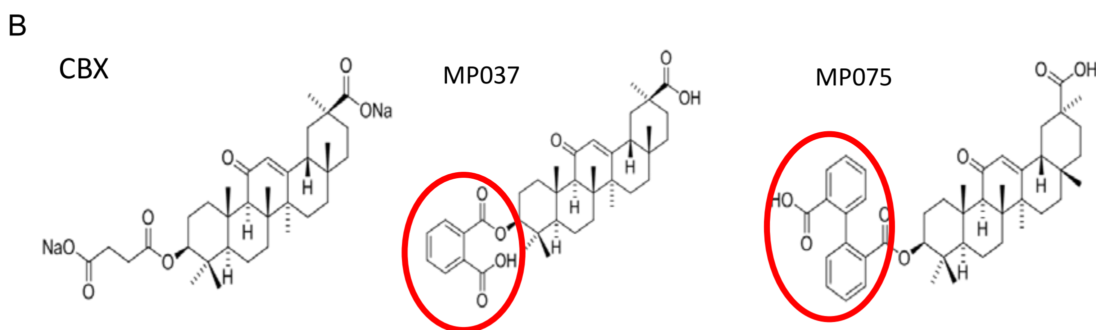

Figure 1: A) Normalized inhibition of the different CBX derivatives tested. Depicted values are normalized to CBX. T-student test was performed to identify statistically significant inhibition differences between each individual derivative vs the original CBX compound. B) 2D schematic representation of different drugs. Red circle highlights the special features.

Up to 13 different CBX derivatives have been successfully tested (**Fig.1A**). Among them, MP37 and MP75 originally stood out. Both compounds shared the feature of incorporating aromatic rings in the long carbon chain preceding the carboxyl (**Fig.1B**). Curiously, MP037 had an unexpected activating effect on the VRAC current (**Fig.1A**). On the other hand, and very interestingly, the MP075 displayed a strong increase in the inhibition of the VRAC current (**Fig.1A**).

Following these very promising results, we and our colleagues decided to perform new derivatives based on the MP037 and MP075 features. To improve their potential clinical use for the treatment of ischaemic insults in the brain, our colleagues incorporated hydroxylamine ester modifications with a double purpose. On one hand, these modifications should increase BBB permeability. On the other hand, once in the brain these modifications could be reverted by hydrolysis. Thus, MP222 and MP228 were obtained from the structures of the MP037 and MP075 respectively. Intriguingly, both MP222 and MP228 displayed similar blocking activity to CBX (**Fig.1A**). However, given that ester modifications are transitory, it is fair to assume a similar behaviour of these compounds to their respective originals (MP037 and MP075).

As the activation of the channel was not the goal of the present project, MP037 and MP222 derivatives were discarded as candidates, and the interesting VRAC activation effect remains to be explored. On the other hand, we kept testing the promising MP228 derivative.

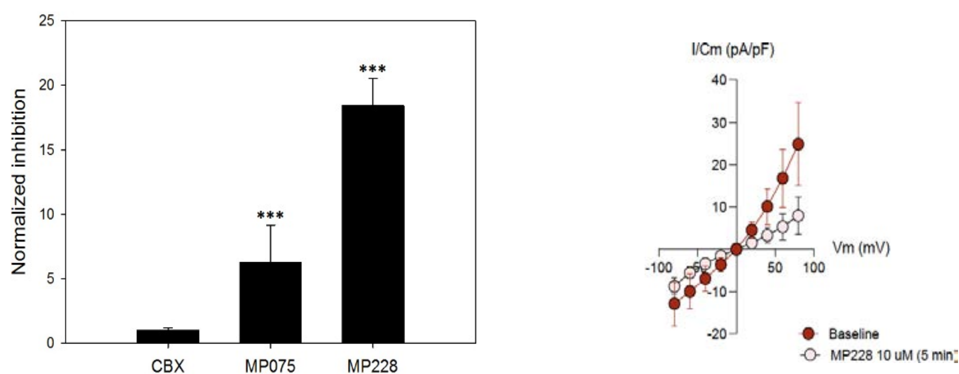

Figure 2. Increased inhibition of MP075 and MP228 vs CBX. At left, experiments in HEK293T LRRC8-/- cells overexpressing LRRC8A+LRRC8E. At right, current-voltage relationship of the VRAC current endogenously expressed in primary astrocytes with or without application of the inhibitor MP228.

MP75 and MP228 were next tested in hek293T *LRRC8 -/-* cell lines overexpressing 8A+8E combination. Interestingly, both MP75 and especially MP228 displayed a huge increase in VRAC inhibition (**Fig.2**). Furthermore, MP228 has also been tested in primary cultured astrocytes and it inhibits the activity of the endogenous VRAC activity present in the cells (**Fig.2**).

DCPIB derivatives

A second set of derivatives testing has been carried out based on the powerful DCPIB VRAC channel blocker. Given the previous experience acquired from CBX derivatives experiments where the introduction of biphenyl groups improved the blocking effect, different derivatives were synthesized and tested (MP349, MP351 & MP399) following that criterion. Unfortunately, none of these compounds improved DCPIB inhibition (**Fig.3A**).

The promising result of DCPIB experiments came from the MP286 derivative. DCPIB synthesis process is hard, specifically due to the presence of a cyclopentane (**Fig.3B-*left***). MP286 greatly simplified the synthesis by changing the cyclopentane by a terbutyl chain (**Fig.3B-*middle***). Furthermore, the MP286 derivative fully retained the inhibition levels of the original DCPIB compound (**Fig.3A**). As indicated previously, another important aspect for the clinical application of this compounds is BBB permeability. For this purpose, ester modifications were introduced in the carboxyl end tip of DCPIB and MP286 obtaining the new derivatives MP277 and MP300 (**Fig.3B-*right***). Both MP277 and MP300 showed reduced inhibition compared to DCPIB

(**Fig.3A**). However, as stated in the previous CBX derivatives section, as ester modifications are expected to hydrolyze, functional activity should not be affected after CNS access. Thus, the optimized synthesis and BBB permeability modifications of the MP300 shaped up this derivative as a good candidate.

From these studies, we decided to focus on two specific compounds, the CBX derivative MP228 and the DCPIB derivative MP300. They were produced in amounts suitable for in vivo experimentation by the MedChem group.

Task 3. Testing one or two inhibitors in a rat model of stroke

We started a collaboration with Anna Planas (CSIC), who is a leading expert in stroke and has already set up in her laboratory the procedure to obtain an ictus in mice after middle artery occlusion. The compounds were injected and the analysis of the effects are under progress. We expect that we will obtain the first results in about two months.

Task 4. Identification of the inhibitor binding site

As mentioned in the intermediate report, the identification in 2018 of the cryo-EM structure of LRRC8A offers the possibility to predict binding sites for VRAC inhibitors and to predict new ones based on docking. Although several cryo-EM structures of LRRC8-VRAC channel have been published, the binding site of the different channel blockers remains unknown. Unravelling the binding site would be important for the designing and optimization of new VRAC channel inhibitors, by making it possible to perform different strategies, for example, virtual docking experiments. Therefore, in the present project many efforts have focused on addressing the identification of the binding site of the VRAC inhibitors (ATP, DCPIB and CBX).

Generation of mutant variants altering blocking sensitivity

Several studies suggest that the pore helix region (and/or nearby residues) of the LRRC8-VRAC channel is involved in the binding of different channel blockers. According to the published structures of the LRRC8A (8A) homohexameric channel, the R103 residue forms the narrowest constriction of the pore (1-4). This feature is also suggested to be shared by the LRRC8D (8D) homohexamer with the equivalent residue F143 (5). Interestingly, the R103F mutation of the essential subunit 8A led to ATP outward current insensitivity in co-expression with the 8C isoform (1). Furthermore, a Cryo EM-structure of the 8A VRAC channel suggested that the R103 constriction is a

key player of the binding site of the DCPIB inhibitor (4). In addition, several studies in Panx1 (a homologous channel with a relatively similar inhibition profile) found that mutation of residues of this same region altered the sensitivity of the channel to different drugs (6,7).

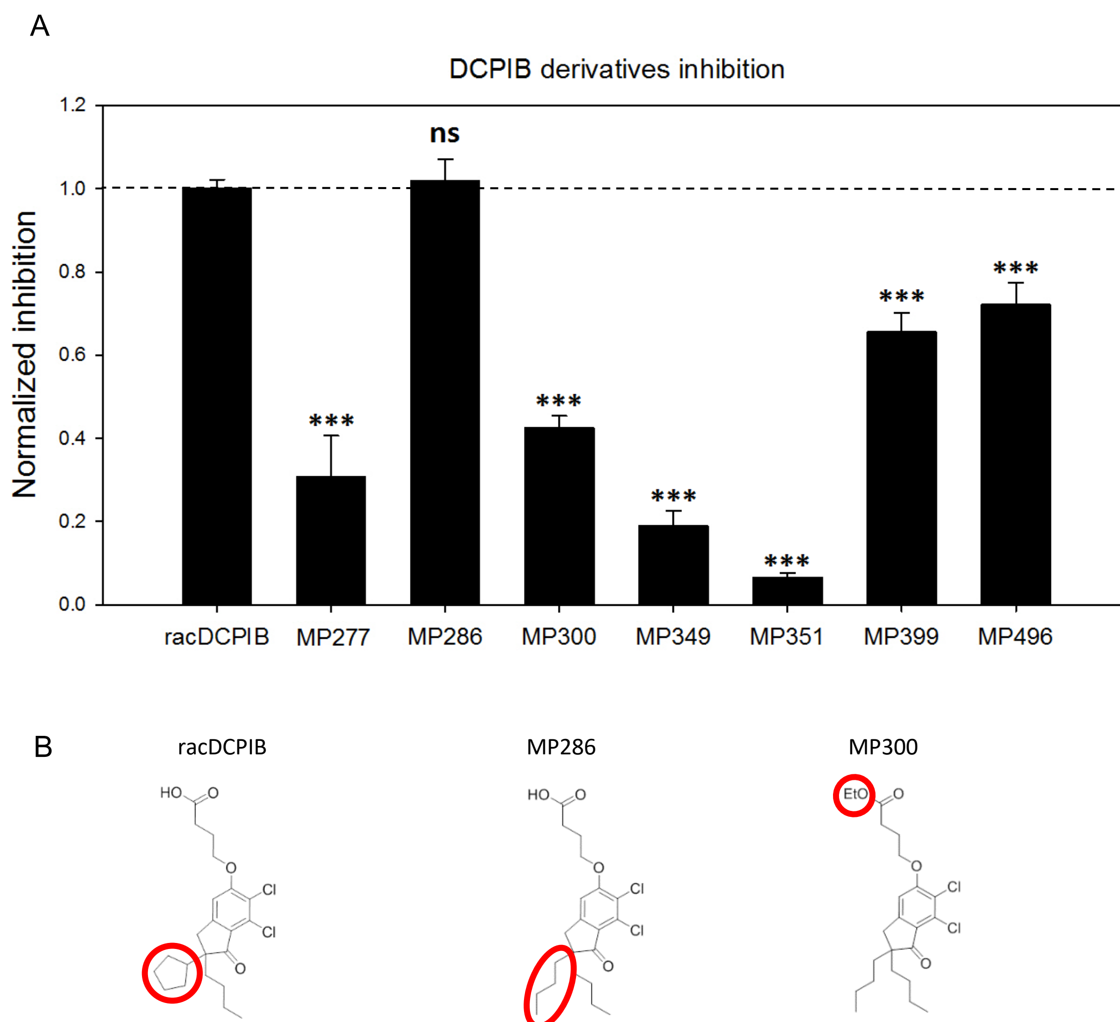

Figure 3: A) Normalized inhibition of the different DCPIB derivatives tested. Depicted values are normalized to DCPIB (racDCPIB). T-student test was performed to identify statistically significant inhibition differences between each individual derivative vs the original DCPIB compound. B) 2D schematic representation of different drugs. Red circle highlights the special features.

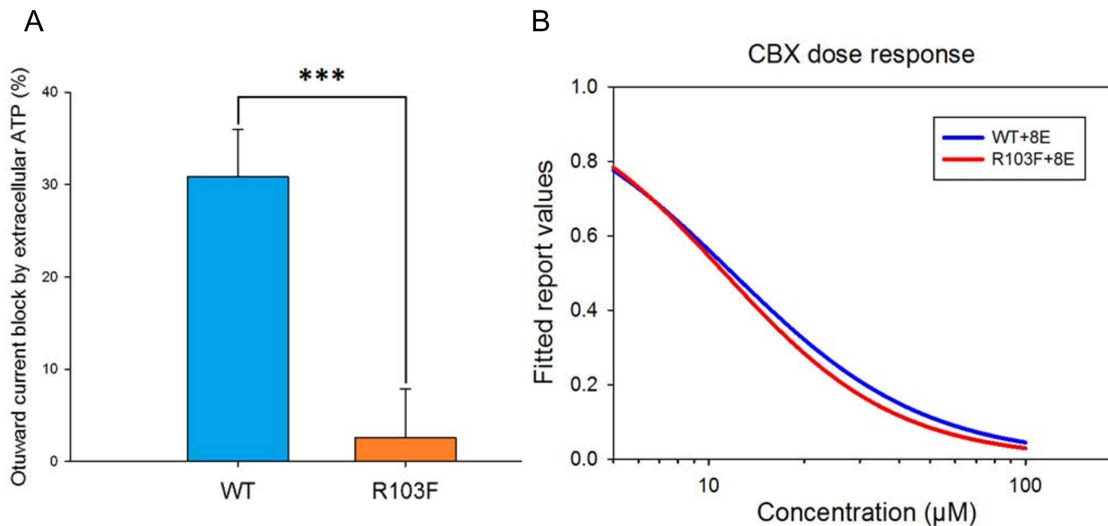

Fig.4: blocking sensitivity of the essential subunit 8A WT/R103F in co-expression with 8E. A) Quantification from TEVC recordings in *Xenopus* oocytes at an extracellular concentration of 2mM ATP. B) Fitted values of the CBX dose-response experiments performed either on the WT 8A (WT) or the R103F 8A mutation (R103F) both in co-expression with 8E. Dose-response experiments were performed at increasing concentrations of CBX in μM: 10, 20, 50, 100.

According to this background, we decided to test whether the R103F 8A mutation could alter blocking sensitivity to different inhibitors. Interestingly, R103F 8A rendered the channel almost insensitive to ATP outward current inhibition in co- expression with the wild-type (WT) 8E subunit in *Xenopus* oocytes (**Fig.4A**). In agreement with previous studies, these results suggested that the R103 residue is critical for the binding of ATP to the LRRC8-VRAC channel. Next, we decided to check for possible effects in the sensitivity of the 8A R103F mutation to the CBX and DCPIB inhibitors. Curiously, neither CBX nor DCPIB inhibition was apparently altered in the R103F 8A mutated VRAC channel (**Fig.4B**). As mentioned before, previous studies suggested that R103 residue could be critical for the binding of DCPIB (4). However, our results in *Xenopus* oocytes strongly suggested that R103 residue was not part of the binding site of this classical VRAC blockers.

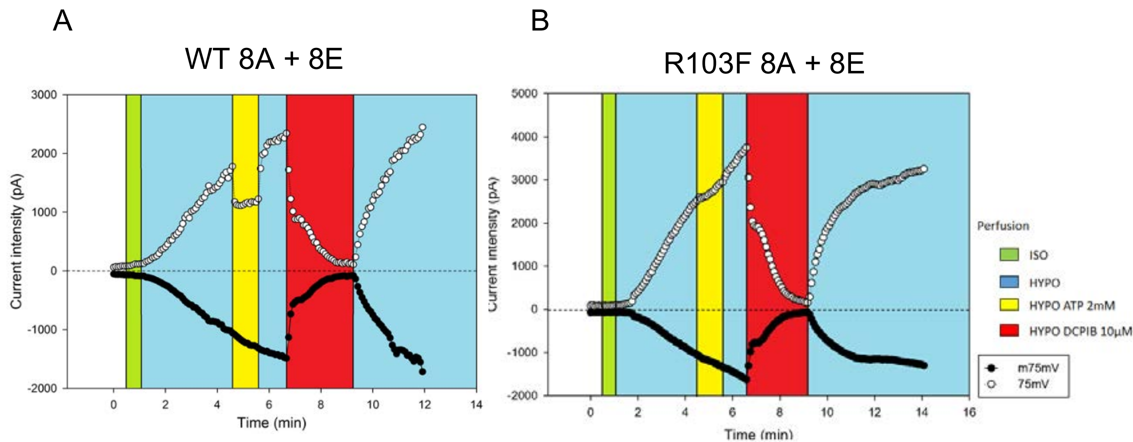

Fig. 5: Representative recordings showing time course of current activity in different bath solutions including: green (isotonic), blue (hypotonic), yellow (hypotonic + 2mM ATP) and red (hypotonic + 10µM DCPIB). Dots correspond to measurements at +75mV (white) and -75mV (black). A) WT 8A + 8E VRAC combination. B) R103F 8A + 8E VRAC combination.

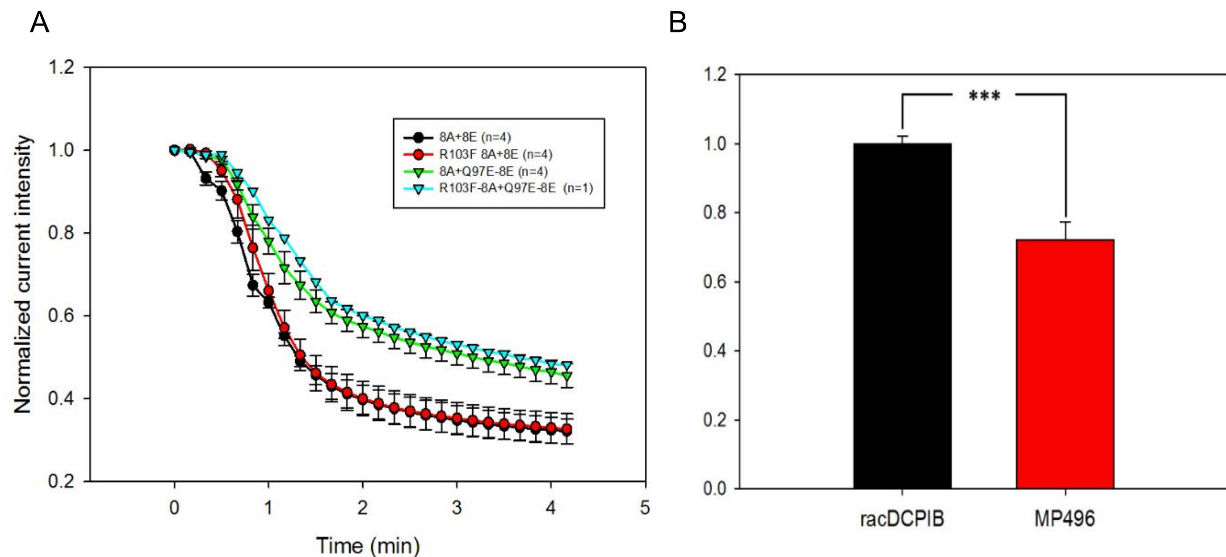

Fig.6: new strategies to unravel the VRAC DCPIB/CBX inhibition binding site. A) Q97E-8E variant diminishes DCPIB sensitivity of the VRAC channel. B) MP496 derivative is a potent inhibitor of the VRAC channel activity. Figure shows normalized values to DCPIB inhibition level (racDPIB).

Given that the *Xenopus* oocytes heterologous system has low levels of endogenous 8A protein (8, 9), we wanted to exclude the possibility that our results were altered by this background. Thus, we decided to carry out similar experiments in hek293T *LRRC8 -/-* cell-lines. With that goal in mind, the post-doctoral researcher Héctor Gaitán-Peñas did a 3-month short stay in the "Istituto di Biofisica – IBF" at Genoa (Italy), learning to perform inhibitors testing and patch clamp experiments at the laboratory of Dr. Michael Pusch. In concordance with oocyte experiments, recordings on hek293T *LRRC8 -/-* cell-lines overexpressing WT/R103F 8A + 8E yielded similar results (**Fig.5A-B**). While WT

8A + 8E outward current was blocked by extracellular ATP (**Fig.5A-*yellow***), R103F mutation led to outward current ATP insensitivity (**Fig.5B-*yellow***). Furthermore, both WT 8A and R103F 8A mutations were similarly inhibited by both DCPIB and CBX inhibitors (compare **Fig.5A-*red*** vs **5B-*red***).

Our results in both oocytes and cell lines strongly suggest that the R103 8A residue is critical for ATP inhibition. On the other hand, no apparent effect could be detected on DCPIB nor CBX sensitivity. Published CEM structures of the channel have been made from homohexamers of the essential subunit 8A (1,2,3,4), or 8D isoform (5).

Furthermore, Panx1 is suggested to have a homoheptameric assembly and thus, blocking sensitivity experiments addressing the binding site have been performed on mutant variants affecting every monomer of the channel (6, 7). The VRAC channel is known to be a heterohexamer formed by different combinations of LRRC8 isoforms. Thus, residues of different isoforms are likely shaping the binding site of the VRAC channel. Recent Panx1 studies identified 2 residues of the pore helix (highly conserved in the LRRC8-VRAC channel) conferring CBX insensitivity when mutated (7). When looking at a homology alignment, these residues correspond to L96 and Q97 in the 8E isoform of the VRAC channel. Interestingly, L96 corresponds to R103 in 8A. Thus, these residues could likely be shaping the narrowest constriction of the heteromeric 8A+8E VRAC channel. Considering this heteromeric nature, it is possible that by mutating only the R103 residue of the 8A subunit we are not significantly altering the shape of the DCPIB/CBX binding site. For all these reasons, we have developed two new mutant variants of the 8E subunit (L96A and Q97E). First results in *Xenopus* oocytes co-expressing these new variants with either WT 8A or R103F 8A, suggest a diminished sensitivity to DCPIB/CBX inhibition (Fig.6A). To confirm these promising results we are currently developing new constructs to test L96A and Q97E 8E variants' sensitivity to CBX/DCPIB inhibition in hek293T *LRRC8 -/-* cell lines. If these results are confirmed, they would be a strong evidence supporting the identification of the DCPIB/CBX inhibition binding site.

Alternatively, we are considering another approach to find the binding site. In collaboration with the Medchem group, they developed the ditopic molecule MP496, which contains an affinity core for the VRAC channel (DCPIB) plus a residue capable of photocrossing on suitable activation by UV light. We were able to test (Fig.6B) that the

photocross analog of DCPIB also inhibits the VRAC channel. Biochemical experiments are in progress.

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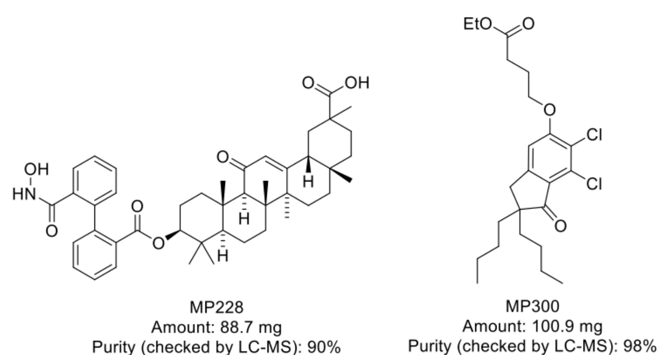
3. Relevance and possible future implications

During the last three years a total number of 20 derivatives have been synthesized. Among them, two specific compounds, MP228 and MP300, have shown a good value of VRAC inhibition in *in vitro* assays, so they have been selected and produced in amounts suitable for *in vivo* experimentation and forwarded for animal testing. Furthermore, we have identified a possible binding site of the inhibitors, which will help in the development of further inhibitors using the available LRRC8 structures.

Compound MP228 is a derivative of carbenoxolone. This new compound incorporates in its structure a diphenyl motif and a hydroxamic acid that replaces the original carboxylic acid of the carbenoxolone. Such a functional group will make it possible for the compound to cross the blood-brain barrier. Compound MP300 is a derivative of DCPIB. In this case, the new derivative does not have chiral centres, thus we have improved its preparation in comparison with the original DCPIB. Moreover, the upper alkyl chain has an ester group replacing the carboxylic acid, which would help the compound to cross the blood-brain barrier.



Ideally, both selected compounds should reach the target VRAC in *in vivo* models and show activity in reducing the glutamate release from astrocytes.

4. Scientific bibliography generated

Publications

1 Review publication. “Chloride channels in astrocytes: structure, roles in brain homeostasis and implications in disease”.

Elorza-Vidal X, Gaitán-Peñas H. Estévez R. Int J Mol Sci 2019 20:1034.

1 publication is in preparation, dealing with the preparation of the less active compounds, their SAR and biological features.

1 publication is in preparation, dealing with structural details of the channel inhibitor binding site.

1 patent is planned regarding the protection of the most active substances and their role in the treatment of ischemia.

1 PhD thesis (Marina Pedrola, Faculty of Pharmacy, Barcelona University, UB) has been fully developed. After passing all the committees she is now writing the final report and the defence is expected to take place around March 22. It will deal with the chemical studies regarding the synthetic approaches and the preparation of the compounds for SAR studies and in vivo testing.