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Mechanism of action of hydroxyl-docosahexaenoic acid molecule in the treatment of Alzheimer's disease: identification of potential receptors in brain

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder very alive in today's society. One of the drugs tested in animals which has shown neuroprotective properties is LP226A1 (a DHA α-hydroxylated derivative).

The aim of this work was focused on the investigation of the mechanism of action of this drug. Specifically, our work was focused on searching for and isolating of LP226A1 binding proteins (LP226BPs) by separation of membrane proteins from mouse cortex by ion exchange (IEX) and hydrophobicity (HIC) chromatography using different sodium chloride (NaCl) concentrations as eluent. The resulting fractions from those techniques were tested in radio-binding assays to LP226A1 in order to test the presence of LP226BPs. Interestingly, our results showed two different protein fractions from IEX with evident binding to LP226A1. One of them was further separated by HIC in order to get LP226BP-enriched fractions. This additional analysis revealed other two fractions which showed binding to LP226A1 and that might be considered as semi-purified LP226BPs.

On the other hand, affinity chromatography was addressed to isolate LP226BPs from soluble protein fractions. For this purpose, the drug was anchored to nitrocellulose. Such anchorage was confirmed by gas chromatography. This LP226A1-bound nitrocellulose was used as stationary phase for affinity chromatography, and indeed, this was incubated with soluble protein from mouse cortex and treated under different conditions in order to promote interaction between anchored LP226A1 with soluble proteins from the medium. Captured proteins were eluted and the resulting samples were analysed by electrophoresis and coomassie blue staining. The results showed that binding of soluble proteins was not via LP226A1, and in fact, they were non-specifically bound to the nitrocellulose membrane.

Finally, western blot analysis was performed to analyse FA2H expression (enzyme in charge of fatty acid α-hydroxylation) in human brain samples from AD patients and healthy controls. These results showed a percentage of AD patients with up-regulated levels of FA2H as compared with healthy controls which might indicate a neuronal response to counteract the early onset of the neuropathology.

RESUM

La malaltia d'Alzheimer és una malaltia neurodegenerativa molt vigent en la societat actual. Un dels fàrmacs estudiats en animals que ha presentat propietats neuroprotectores és el LP226A1 (un derivat α-hidroxilat del DHA).

L'objectiu d'aquest estudi es va enfocar en la investigació del mecanisme d'acció d'aquest fàrmac. Concretament, la nostra tasca es va centrar en la recerca i aïllament de proteïnes d'unió al LP226A1 (LP226BPs) mitjançant el fraccionament de les proteïnes de membrana de còrtex cerebral de ratolí mitjançant tècniques de cromatografia d'intercanvi iònic (IEX) i de hidrofobicitat (HIC) utilitzant diferents concentracions de clorur sòdic (NaCl) com eluent. Les fraccions resultants van ser analitzades mitjançant assaigs d'unió al radio-lligand LP226A1 amb la finalitat de detectar LP226BPs. Aquests assajos van mostrar LP226BPs en dues fraccions obtingudes per IEX. Una d'elles va ser posteriorment fraccionada mitjançant HIC per obtenir mostres enriquides en LP226BPs. Aquest anàlisis addicional va mostrar dues fraccions més amb LP226BPs i que es podrien considerar com LP226BPs semi-purificades.

Per altra banda, també es va dissenyar un assaig de cromatografia d'afinitat per aïllar LP226BPs de mostres de proteïna solubles. Amb aquest propòsit, el fàrmac es va absorbir sobre nitrocel·lulosa. Aquest anellatge es va confirmar mitjançant cromatografia de gasos. La nitrocel·lulosa amb LP226A1 unit es va usar com a fase estacionària per cromatografia d'afinitat, i es va incubar amb proteïnes solubles de cervell de ratolí sota diferents condicions per tal d'afavorir la interacció entre el LP226A1 ancorat i les proteïnes solubles del medi. Les proteïnes capturades van ser eluïdes i les mostres obtingudes van ser analitzades mitjançant electroforesi i tenyides amb blau de coomassie. Els resultats van mostrar que la unió d'aquestes proteïnes no era a través de LP226A1 sinó que realment estaven unides no-específicament a la membrana de nitrocel·lulosa.

Finalment també es van fer assajos de Western Blot per analitzar l'expressió de la FA2H (enzim responsable de la α-hidroxilació d'àcids grassos) en mostres de cervell de pacients d'Alzheimer i controls sans. Els resultats van mostrar un percentatge dels pacients afectats per la malaltia amb alts nivells de FA2H en comparació amb els controls sans el que podria indicar una resposta neuronal per contrarestar l'aparició prematura de la neuropatologia.

RESUMEN

La Enfermedad de Alzheimer (EA) es una enfermedad neurodegenerativa muy vigente en la sociedad actual. Uno de los fármacos estudiados en animales que ha presentado propiedades neuroprotectoras es el LP226A1 (un derivado α-hidroxilado del DHA).

El objetivo de este trabajo se centró en la investigación del mecanismo de acción de éste fármaco. En particular, nuestro trabajo se ha centrado en la búsqueda y aislamiento de proteínas de unión a LP226A1 (LP226BPs) realizando el fraccionamiento de las proteínas de membrana de corteza cerebral de ratón mediante técnicas de cromatografía de intercambio iónico (IEX) y de hidrofobicidad (HIC) utilizando diferentes concentraciones de cloruro sódico (NaCl) como eluyente. Las fracciones resultantes fueron analizadas en ensayos de unión al radio-ligando LP226A1 con la finalidad de detectar LP226BPs. Estos ensayos mostraron LP226BPs en dos fracciones obtenidas por IEX. Una de ellas se fraccionó por HIC para obtener muestras enriquecidas en LP226BPs. Este análisis adicional mostró otras dos fracciones con LP226BPs y que se podrían considerar como LP226BPs semi-purificadas.

Por otra parte, también se diseñó un ensayo de cromatografía de afinidad para aislar LP226BPs de muestras de proteína solubles. Con este propósito, el fármaco se adsorbió sobre nitrocelulosa. Este anclaje se confirmó mediante cromatografía de gases. La nitrocelulosa con LP226A1 unido se empleó como fase estacionaria para cromatografía de afinidad, y de hecho, se incubó con proteínas solubles de cerebro de ratón bajo diferentes condiciones para favorecer la interacción entre el LP226A1 anclado y las proteínas solubles del medio. Las proteínas capturadas se eluyeron y las muestras obtenidas se analizaron mediante electroforesis y tinción con azul de Coomassie. Los resultados mostraron que la unión de estas proteínas no era a través de LP226A1 sino que estaban unidas no-específicamente a la membrana de nitrocelulosa.

Finalmente también se hicieron ensayos de Western Blot para analizar la expresión de la FA2H (enzima responsable de la α-hidroxilación de ácidos grasos) en muestras de cerebro de pacientes de EA y controles sanos. Los resultados mostraron un porcentaje de los enfermos de EA con altos niveles de FA2H en comparación con los controles sanos lo que podría indicar una respuesta neuronal para contrarrestar la aparición temprana de la neuropatología.

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INTRODUCTION

Alzheimer Disease (AD) is a neurodegenerative disorder that affects around 35.6 million people around the world ("Informe Mundial sobre el Alzheimer," 2009).

Firstly, only seems to be simple oversights and humour changes but as time goes on, the AD symptoms are more detectable and the disease process erases all the patient memories, causes motor dysfunction and several cognitive impairment (dementia).

The most detachable neuropathological characteristic of the AD brain are macroscopic lesions such as cortical atrophy and synaptic (and neuronal degeneration), and microscopic lesions such as is the presence of extracellular amyloid plaques and intraneuronal neurofibrillary tangles (NFT). The soluble building blocks of these structures are amyloid-β peptides (Aβ) for plaques and the tau protein for tangles. (Bloom, 2014).

Changes in the intraneuronal development of the cytoskeleton are caused by abnormally phosphorylated tau protein. The intraneural material produces the NFT and neuropil threads (dystrophic neurites; NT). The spread of these intraneuronal changes is not uniform. NFT and NT display a selective connexion of specific architectonic units that follow a predictable sequence in the AD development. Based on the NFT and NT development, six stages can be differentiated. There is a linear correlation between the six stages of the brain destruction and the intellectual stage evaluations of the patients.

In transentorhinal stages I and II, NFT and NT are slowly developed at their predilection sites, nevertheless the brain changes remain below the threshold which produces clinical symptoms.

Limbic stages III and IV are associated at subtle personality changes and a deficiency of cognitive functions. That stages do not show microscopically detectable atrophy and the main part of neocortex is not affected. Most detectable changes are located at few allocortical regions. Specifically, in Stage III NFTs develop and accumulate in limbic structures such as the subiculum of the hippocampal formation and the stage IV the NFTs accumulate at amygdala, thalamus and claustrum. (Serrano-pozo, Frosch, Masliah, & Hyman, 2011)

Neocortical stages V and VI are associated at the final stages of the AD. They are associated at high levels of NFT and NT in all subdivisions of cerebral cortex. In addition, those stages are related at severe destruction of neocortical association areas. (Braak & Braak, 1995)

Nowadays, most Alzheimer patients are treated with different drugs that are focused in avoiding the cognitive symptoms of the disease. There are mainly two types of drug: cholinesterase inhibitors and NMDA receptor antagonists. The cholinesterase inhibitors (donepezil, galantamine and rivastigmine) prevent the acetylcholine decomposition at synaptic densities and elevate the levels of this neurotransmissor which are abnormally low in the AD brain because of cholinergic neuron degeneration. On the other hand, NMDA receptors antagonists work regulating the glutamate signalling at synaptic terminals. Aβ peptide is a NMDA receptor antagonist which induces neuronal death by excitotoxicity. This drug prevent this kind of Aβ-mediated neurotoxicity. Nevertheless, all those drugs do not halt the progression of the disease and they are efficient only for a reduced percentage of patients. They help to slow down the rate at which the disease gets worse during a short period of time (normally a few months) (Tayeb, Yang, Price, & Tarazi, 2012).

Previous Results

This disease has been associated with a decline of docosahexanoic acid (DHA) levels. DHA is a polyunsaturated omega-3 fatty acid with 22 carbon atoms that is present in the entire organism but the major part is concentrated in the brain. In this context, DHA has been tried as a possible treatment of the disease in human trials, even so that treatment only show positive results in a subgroup of moderately affected patients (Torres et al., 2014). With the aim of obtaining positive results in a higher percentage of affected patients, our research group designed and synthesized an hydroxyl-derivative of DHA show one hydroxyl group at the alpha carbon turning the DHA into a 22:6 fatty acid named as 2-hydroxydocosahexaenoic acid (OHDHA). This molecule was patented by Lipopharma Therapeutics S.L. under the name of LP226A1. Interestingly, 2-hyodroxilation occurs during de novo ceramide synthesis and is catalysed by fatty acid 2-hydroxilase (FA2H). This enzyme is highly expressed in brain tissue and its deficiency has been associated with a form of neurodegeneration with brain iron accumulation (Kruer et al., 2010).

Several studies demonstrated that the 2-hydroxyl group in sphingolipids has a profound effect in the lipid organization within membranes because of its hydrogen bonding capacity (Alderson et al., 2004). Specifically, in recent preclinical studies, LP226A1 has shown a potent effect regulating membrane lipid composition and structure and, in addition, the administration of this compound leads to cognition improvement in AD transgenic mouse models. Moreover, this compound dowregulates Aβ accumulation and Aβ-induced tau protein hyperphosphorylation, so that LP226A1 promotes neuroprotection and cell survival against different known AD-associated insults. In addition, this compound can also induce neuron stem cell proliferation. However, the molecular and cellular mechanism behind these effects remain still largely unknown (Torres et al., 2014) (Fiol-Deroque et al., 2013).

AIMS

The aforementioned molecule LP226A1 is a novel therapeutic candidate for the treatment of the AD-related neurodegeneration. However, the mechanism of action of this compound is still largely unknown. In this sense, when poly unsaturated fatty acids (PUFAs) enter at the body through the diet, they interact with the gastrointestinal tract. Absorption and body distribution of fatty acids is influenced by their structure. (Bordoni, Di Nunzio, Danesi, & Biagi, 2006) When fatty acids get into brain they interact with cell membrane and, in actually, they are incorporated into them. For this reason, the present study has been focused on searching for LP226A1 binding proteins (LP226BPs) in mouse cerebral cortex. Even though our experiments were focused on membrane proteins, it is noteworthy that the soluble protein fraction obtained from brain might also contain fatty acid receptors. Therefore, an attempt of affinity chromatography was designed and assayed in order to detect LP226BP in theses samples.

On the other hand, since previous results have demonstrated that LP226A1 (which is a 2-hydroxy fatty acid), has a potential neuroprotection activity (Fiol-Deroque et al., 2013) (Torres et al., 2014), we have also focused our research on investigating FA2H enzyme expression in AD patient brains. This enzyme catalyses the synthesis of 2-hydroxy fatty acids (NCBI, 2015), therefore knowledge about FA2H expression levels in the human brain would help us to understand the role-of α-hydrosylated fatty acids in AD pathophysiology.

To sum up, the aims of the present are:

-Brain membrane protein separation by chromatographic techniques such as ion exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC) in order to isolate LP226BPs from obtained chromatographic fractions.

-Designing of affinity chromatography technique using the soluble protein fraction obtained from mouse brain as sample.

-Analysis of FA2H enzyme expression in human brain samples from AD patients and controls with no neuropathological lesions.

MATERIAL AND METHODS

1. Sample preparation

1.1. Sample preparation for Ion Exchange Chromatography

1.1.1. Materials and Reagents

-**Dounce's homogenizer**
-**Mouse brain cortex**
-**Hypotonic buffer:** 1mM EDTA, 1mM EGTA, 10mM Tris-HCl pH 7.4
-**Solubilisation buffer:** 1% (w/v) CHAPS, 1mM EDTA, 1mM EGTA, 20mM Tris-HCl pH 8.1
-**Complete Protease inhibitor cocktails** (Roche)

1.1.2. Method principle

Cerebral tissue is mechanically disrupted in hypotonic buffer at physiological pH in order to maximize cell lysis. Cellular membrane content is isolated by differential centrifugation and then resuspended in solubilisation buffer containing a complete cocktail of protease inhibitors. This buffer contains CHAPS which is a zwitterionic detergent that allows gentle protein solubilisation (non-denaturing conditions) and is not ionically charged at pH 8.1. Most of cellular proteins show isoelectric point below 7.5 so that most of cellular proteins show net negative charge at pH 8.1. Non-denatured and negatively charged proteins will bind to a positively-charged resin during ion-exchange chromatography (see section ***2***).

1.1.3. Procedure

a) Cerebral cortex from two mice were dissected and homogenized in cold hypotonic buffer in presence of a complete cocktail of protease inhibitors at a ratio 20:1 (buffer:tissue) by Dounce's.
b) The resultant supernatant was centrifuged at 1000xg for 5 minutes at 4ºC in order to precipitate nucleus and debris.
c) Supernatant was centrifuged at 40.000xg for 30 minutes at 4ºC to separate total membrane fraction from the soluble protein fractions.
d) Pellet was resuspended in 2 ml of solubilisation buffer containing a complete cocktail of protease inhibitors and incubated for 60 minutes at 4ºC to allow membrane protein solubilisation.
e) Solubilised membranes were centrifuged at 40.000xg for 30 minutes at 4ºC to separate CHAPS-solubilised proteins from non-solubilised proteins (pellet). The resultant supernatant constitutes the sample for IEX.
f) Protein concentration was determined at 280nm (λ) (Nanodrop spectrophotometer; Thermo Fisher).

1.2. Sample preparation for western blot

1.2.1. Materials and reagents

-**Human brain hippocampus**
-TriPure Isolation Reagent: monophasic solution of phenol and guanidine thiocynate for RNA, DNA and protein isolation
-Chloroform (molecular biology grade)
-Isopropanol (molecular biology grade)

-Ethanol absolute (HPLC grade)
-Diethylpyrocarbonate (DEPC-treated RNase-free water).
-Protein wash solution (0.3M guanidine in 95% ethanol)
-Urea solution (8M Urea, 4% SDS, 20mM Tris-HCl pH 7.5)
-Loading buffer 10X (also called Laemli's buffer): 0.025%(w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1% (w/v) SDS, 20mM Tris-HCl pH 6.8

1.2.2. Method principle

Different biomolecules are separated according to their affinity for aqueous and organic solvents. Then proteins are precipitated by 2-propanol and washed before solubilisation in 4% SDS and 8M Urea. These protein samples are denatured and display net negative charge.

1.2.3. Procedure

a) A fragment of human hippocampus weighting 50-100 mg were mixed with 0.5 ml Tripure Isolation Reagent and homogenized by Polytron.
b) Each homogenized sample was incubated for 5 minutes at room temperature to ensure the complete dissociation of nucleoprotein complexes.
c) 0.1 ml of chloroform were added to each sample and shacked vigorously at least for 15s.
d) To separate the solution into three phases, the tube was centrifuged at 12.000xg for 15 minutes at 4ºC.
e) The sample was separated into three phases: the upper (aqueous) phase containing RNA and the interphase and lower (organic) phase containing protein and DNA. Since it has been widely described that RNA isolated from post-mortem tissue shows very low quality the decision of discarding this material was made for the present work.
f) 0.15 ml of 100% ethanol were added at interphase and lower phase. The resultant was centrifuged at 2.000xg for 5 minutes at 4ºC to precipitate DNA.
g) Isopropanol (1.35 ml) was added to the remaining interphase-organic phase. Samples were incubated for 15 minutes to allow protein precipitation.
h) Proteins were precipitated by centrifugation at 12.000xg for 15 minutes at 4ºC.
i) Protein pellet was dried under vacuum and resuspended in 500µl of urea/SDS solution and incubated overnight with gentle shaking at room temperature to allow protein solubilisation.
j) Protein concentration was determined by Lowry's method.
k) Each sample was adjusted to a final concentration of 2 mg/ml by adding the required volumes of 10X-concentrated loading buffer and solubilisation buffer.

2. Ion Exchange Chromatography (IEX)

2.1. Material and Reagents

-**Fast Performance Liquid Chromatography** (FPLC). AKTA Purifier (GE Gealthecare).
-Resource Q (1ml) column
-HiTrap Desalting (5ml) column
-Solubilisation buffer: 1% (p/v) CHAPS, 1mM EDTA, 1mM EGTA, 20mM Tris-HCl pH 8.1
-Elution buffer: 1% (p/v) CHAPS, 1mM EDTA, 1mM EGTA, 1M NaCl, 10Mm Tris-HCl pH 8.1
-**Desalting buffer**: 1% CHAPS, 1mM EDTA, 1mM EGTA, 140mM NaCl, 20mM Tris-HCl pH 8.1

2.2. Method principle

IEX is used to separate proteins that have differences in their charge properties. So, different proteins will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge. Each protein has its own unique net charge versus pH relationship.

The IEX medium used for this study comprises a matrix of spherical particles substituted with ionic groups that are positively charged (anion exchange chromatography). The proteins which bind are effectively concentrated onto the column while proteins that do not have the correct surface charge pass through the column, eluting during or just after sample application. Then, proteins are eluted by increasing the ionic strength (salt concentration) of the buffer. As ionic strength increases, the salt ions compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. (Walton, 1968).

2.3. Procedure

Before applying buffers to the column, all of them have to be filtered through 0.22 µm filters. In addition, to avoid formation of air bubbles into the column, all buffers have to be degasified by ultrasound application.

-The column was equilibrated with the solubilisation buffer for 10-20 column volumes (CVs).
-The sample (2ml) (see section ***1.1***) was applied to the column and a posterior wash with 8 CVs was applied with starting buffer.
-Step salt elution was performed with 5 CVs of each NaCl concentration (100mM, 200mM, 300mM, 400mM and 500mM)
-Finally, the column was washed with 5 CVs of 1M NaCl to elute any remaining ionically-bound material and re-equilibrated with 3 CVs of starting buffer.

Eluted proteins were collected into 1ml fractions in Eppendorf tubes.

Desalting procedure

Desalting procedure (also named buffer exchange) was performed for 1M-NaCl-eluted protein fraction following supplier instructions, briefly:
-The desalting column (containing Sephadex G25 beads, GE Healthcare) was equilibrated with desalting buffer until conductivity and pH baselines were stable.
-The sample was applied to the column (1ml, 1 chromatographic fraction). While this application, the eluted liquid was discarded.
-The protein was eluted with 1ml of desalting buffer and collected.
-Finally, the column was equilibrated with desalting buffer (until conductivity baseline become stable).

3. Hydrophobic interaction chromatography (HIC)

3.1. Material and reagents

-Fast Performance Liquid Chromatography (FPLC). AKTA Purifier (GE Gealthecare).
-HiTrap phenyl Fast Flow column (1ml) (GE Healthcare)
-**Sample**: 300-mM-eluted protein fraction from previous IEX (1% (w/v) CHAPS, 1mM EDTA, 1mM EGTA, 20mM Tris-HCl pH 8.1
-Sample HIC Buffer: 1mM EDTA, 1mM EGTA, 5M NaCl, 20mM Tris-HCl pH 7.0
-HIC starting buffer: 0.5% (w/v) CHAPS, 1mM EDTA, 1mM EGTA, 2.6M NaCl, 20mM Tris-HCl pH 7.0
-HIC elution buffer: 0.5% (w/v) CHAPS, 1mM EDTA, 1mM EGTA, 20mM Tris-HCl pH 7.0
-HIC final elution buffer: 1% (w/v) CHAPS, 1mM EDTA, 1mM EGTA, 20mM Tris-HCl pH 7.0

3.2. Method principle

HIC was performed after elution in with IEX in order to perform further separation of one specific protein fraction obtained by IEX. Proteins in aqueous solution have various surface residues exposed to solvent to different degrees, generally, hydrophobic groups are located internally in the protein 3D structure but some are exposed. Sample hydrophobic components interact with hydrophobic functional groups of the column and are retained according to them net hydrophobic level.

In this case, the column contained a matrix with hydrophobic groups consisting on phenyl groups (covalently bound to the resin via ether bonds) which binds proteins from aqueous solutions to different extends depending on the protein structure. During HIC, sample proteins bind to the column in presence of high concentration of NaCl (without inducing protein precipitation). Under these conditions, hydrophobic interaction is favoured because protein hydrophobic groups are more accessible from the aqueous medium. Protein elution is performed by decreasing the salt concentration stepwise. (GEHealthcare, 2012)

3.3. Procedure

The sample comes from IEX elution with 300mM NaCl. Sample composition was modified to increase NaCl concentration up to 2.6M. For this purpose, 2.5ml of 300mM NaCl IEX eluted sample were mixed to 2.5ml of Sample HIC Buffer and the pH was adjusted at 7.0. The resultant sample (5 ml) contained protein peak from IEX, 0.5% (w/v) CHAPS, 1mM EDTA, 1mM EGTA, 2.6M NaCl and 20mM Tris-HCl pH 7.0.

Before applying buffers at the column, all of them have to be filtered through 0.22µm filters and degasified by ultrasound application.

-The column was equilibrated with the HIC starting buffer for 20 CV.
-Sample (5 ml) was applied to the column that was washed with 10 CVs of HIC starting buffer.
-Step salt elution consisted in applying 5 column volumes of each salt concentration (2M, 1.5M, 1M NaCl, 500mM and 140mM NaCl)
-Finally, the column was washed with 5 column volumes of HIC final elution buffer (140mM NaCl, 1% (w/v) CHAPS) to elute any remaining bound material and re-equilibrated with 3 column volumes of starting buffer.
Eluted proteins with each step of the NaCl discontinuous gradient were collected into 1ml fractions in 1.5ml Eppendorf tubes.

4. Radio-Binding Assay

4.1. Material and reagents

For IEX chromatography samples

-**Glass fibre filter** (Whatman GF/B)
-Polyethyleneimine 0.3% (w/v)
-Semi-Auto Harvester, 4x12 filter grid (Brandel)
-Vacuum oil pump (Edward's nº 5)
-Scintillation liquid (Perkin Elmer)
-Scintillation counter (Beckman Coulter)

-Samples were obtained from IEX chromatography

-**Binding reaction for IEX**: 0.3ml sample, 0.3% (w/v) CHAPS, 2µM ^{3}H-LP226A (Moravek Biochemicals, USA), 1mM EDTA, 1mM EGTA, 140mM NaCl, 50mM Tris-HCl pH 7.5, (and 1mM cold LP226A1 just for non-specific reactions)

-Reaction base buffer for IEX: 4µM ^{3}H-LP226A, 1.4 µM EDTA, 1.4 µM EGTA, 84mM Tris-HCl pH 7.3

-**Samples were obtained from HIC chromatography**

-**Binding reaction for HIC**: 0.3ml sample, 0.3% (w/v) CHAPS, 2µM ^{3}H-LP226A (Moravek Biochemicals, USA), 1mM EDTA, 1mM EGTA, 2.5M NaCl, 50mM Tris-HCl pH 7.6, (and 1mM cold LP226A1 just for non-specific reactions)

-Reaction base buffer for HIC (2X): 4µM ^{3}H-LP226A, 1.4 µM EDTA, 1.4 µM EGTA, 83mM Tris-HCl pH 7.6

4.2. Method principle

The binding assay is used to detect which protein fraction obtained from IEX or HIC contains LP226BPs. Samples used for this procedure are those containing the protein peak eluted after each salt-concentration step. This methodology is based on sample protein interaction with polyethileneimine-soaked glass fibre filters. When binding between ^{3}H-LP226A1 and membrane proteins appears, the radioactive molecule remains retained to the filter as well. Therefore, filter-retained radioactive signal can be quantified.

Nevertheless, ligand-receptor binding can be specific or non-specific. To distinguish that, the same reaction is reproduced twice: with and without an excess of cold ligand (non-tagged LP226A1). These reactions are named non-specific binding and total binding reactions, respectively. The aim of this excess of cold ligand would be displacing the specific binding of the tritium-tagged ligand from one particular receptor. When radioactivity signal detected in both reactions (with the same sample) is the same, it means that no specific binding appeared between sample proteins and ^{3}H-LP226A1. While if detected signal is higher in total binding reaction as compared with non-specific binding reaction, it means that specific binding takes place between sample proteins and ^{3}H-LP226A1. *(**Fig. 1**).*

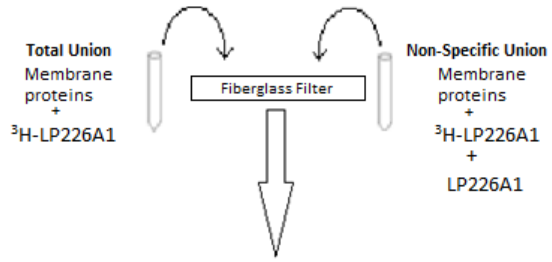

Fig. 1. Representative Scheme of the protein interaction with LP226A1. *On the left it is represented the total binding reaction in which the membrane proteins are bounded to ^{3}H-LP226A1. On the right it is represented the non-specific reaction where cold ligand (LP226A1) is added.*

4.3. Procedure

Any procedure using radioactively tagged molecules was performed at the Radioactivity Facilities of the University of Balearic Islands (facility reference: IR-697) under the supervision of Mª Trinidad García Barceló (facility supervisor) after receiving the basic training course that allows access to those facilities.

-All the reagents of specific and non-specific binding reactions were incubated 30 minutes at 25ºC.
-Polyethyleneimine-soaked glass fibre filters were placed at the harvester system. *(**Fig. 2**)*
-To stop reactions, 5 ml of cold wash buffer (4ºC) were added to each tube and immediately transferred to the filters.
-Filters were washed with 5 ml of cold wash binding buffer three times and, removed from the Harvester System.
-When filters were dried, they were transferred into scintillation vials with 3 ml of scintillation liquid for being analysed.

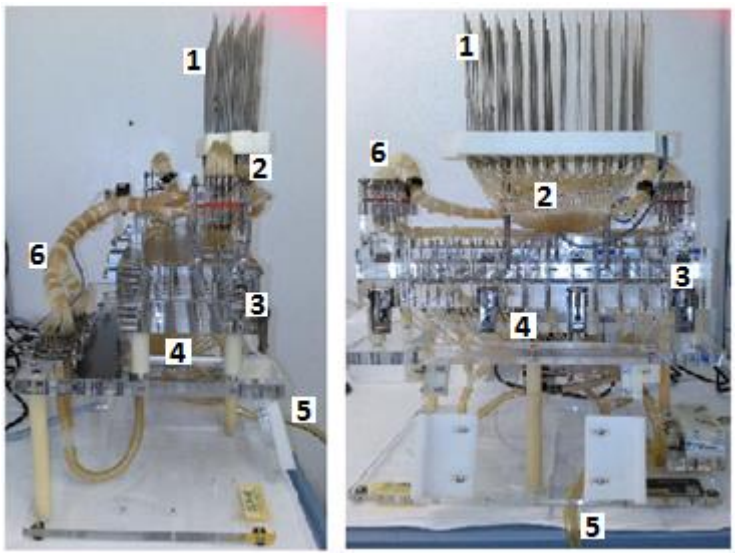

Fig. 2. Brandel Harvester System. *Metallic needles (1) were inserted into test-tubes containing the specific and non-specific binding reactions. Firstly, 5ml of cold wash buffer were added to each tube through metallic needles. Secondly the system collected the samples and transferred them by thin plastic tubes (2) to the fiberglass filters that were located inside the transparent block (3). When sample was filtrated, it was collected by other thin tubes (4) that joined at a big one (5) which was derived to a waste bottle. Cold wash buffer entered at the system via the tubes marked with number 6.*

5. Anchorage of LP226A1 to nitrocellulose support

5.1. Testing conditions

5.1.1. Material and Reagents

-Five 1-cm^2 pieces of nitrocellulose membranes

-Drug-Buffer A: 60mM LP226A1 in 5% (v/v) ethanol

-Drug-Buffer B: 60mM LP226A1 in 1% (w/v) CHAPS, 140mM NaCl, 20mM Tris-HCl pH 7.5

-Drug-Buffer C: 60mM LP226A1 in TTBS

-**TTBS:** 0.1% (w/v) tween20, 140mM NaCl, 20mM Tris-HCl pH 7.5

-Buffer B: 1% (w/v) CHAPS, 140mM NaCl, 20mM Tris-HCl pH 7.5

5.1.2. Method principle

Affinity chromatography separates molecules based on the reversible interaction between target protein and one specific ligand attached to a chromatography matrix (stationary phase) (Cuatrecasas & Anfinsen, 1971).

In the present work nitrocellulose is proposed as a possible stationary phase for affinity chromatography. Nitrocellulose membranes shown a binding affinity for many substrates including lipids (or fatty acids). This is because in the present work we have used this support for LP226A1.

5.1.3. *Procedure*

1. One-cm^2 pieces of nitrocellulose membrane were incubated for 10 minutes under different conditions:
   a) Membrane 1 + 1ml of Drug buffer A
   b) Membrane 2 + 1ml of Drug buffer B
   c) Membrane 3 + 1ml of Drug Buffer C

2. Each membrane piece was dried at room temperature for 5 minutes and washed with the corresponding drug buffer (without LP226A1).
   a. Membrane 1. Three washes in 5% ethanol
   b. Membrane 3. Three washes in Buffer B.
   c. Membrane 4. Three washes in TTBS

3. Nitrocellulose membrane pieces were dissolved in 1ml of methanol and mixed with 2ml of chloroform and 0.75 ml 0.9% NaCl solution. Lipid extraction and derivatization for gas chromatography was performed to check if LP226A1 was bound to nitrocellulose membrane:
   a) Sample 1. Dried membrane 1
   b) Sample 2. Dried membrane 2
   c) Sample 3. Dried membrane 3

5.2. Gas Chromatography

5.2.1. *Material and Reagents*

-**Gas chromatographer:** Agilent GC7890A with autosampler 7693
-Column: Supelco SP-2330, 30m x 320µm x 0.2µm
-Chloroform (HPLC grade)
-Methanol (HPLC grade)
-0.9% (w/v) NaCl solution
-Hexane (HPLC grade)
-CH_3OH:acetylchloride (10:1, v:v)

5.2.2. *Method principle*

Gas chromatography was used to check if the LP226A1 was bound to the nitrocellulose membrane. Fatty acids are not volatile, so that it is necessary to perform lipid derivatization consisting in transmethylation and trimethylsilylation to make these molecules volatile.

Gas chromatography is a technique for separating chemical substances that relies on differences in partitioning behaviour between a following mobile phase and a stationary phase to separate the components in a mixture. The method consists of introducing the test sample into a stream of an inert gas that acts as carrier. The stream gas is passed through the packed column, through which the components of the sample move at velocities that are influenced by the degree of interaction of each constituent with the stationary non-volatile phase. The substances having the greater interaction with the stationary phase are retard to a greater extent and consequently separate from those with smaller interaction. As the components elute from the column they

can be quantified by a detector (The Lynde Group, 2015). In each sample, a known amount of margaric acid is added like an internal standard which allow precise quantification of the target molecule.

5.2.3. Procedure

a) Extraction of the LP226A1 from the nitrocellulose membrane

-Each piece of nitrocellulose membrane (see section ***5.1.3***) was dissolved in 1ml of methanol and mixed with 2ml of chloroform and 0.75ml of saline solution (0.9% NaCl).
-The resulting solution was centrifuged at 1000xg for 10 minutes to separate organic and aqueous phases. Nitrocellulose accumulated and compacted at the interphase. The organic phase containing LP226A1 was collected.
-Margaric acid (C17:0; 50pmoles) was added to each tube like an internal standard for gas chromatography. The organic phase was evaporated under argon flow.
-Lipid film was resuspended in 166µl of hexane and 3ml CH_3OH:acetylchloride (10:1, v:v) were added (Christie, 2011) to allow fatty acid transmethylation.
-The sample was incubated at 100ºC for 90 minutes under inert atmosphere in Pyrex screwed-capped tubes (Morrison & Smith, 1964).
The products obtained from the transmethylation step consist of fatty acid methyl esters (FAME) which were separated after three consecutive extractions in hexane.
-3 ml of H_2O and 1 ml hexane were added to the transmethylation reaction product.
-The mixture was vortexed and centrifuged at room temperature at 1000xg for 5 minutes. During that centrifugation a phase separation occurred.
-The upper phase (hexane) containing FAMEs was collected and remaining volume was washed twice with another 1 ml of hexane.
-The collected hexane phases were mixed, evaporated under argon flow and resuspended in 1ml of hexane.
-Hydroxyl fatty acids are not completely volatile in their methyl ester form, so a second derivatization consisting of an addition of a trimethylsilyl group to each hydroxyl residue was performed. (Alderson et al., 2004)
-For such derivatization FAMEs sample was evaporated under argon flow. The resultant lipid film was resuspended in 200µl of N_2O-bis (trimethylsilyl acetamide and heated in a capped vial at 76ºC.
-The solvent was evaporated under argon flow and the pellet was resuspended in 1ml of hexane prior to gas chromatography assay.

The chromatography was run with a 60:1 split. The injector and the detector temperature was 250ºC. The initial temperature was 130ºC and it was maintained for 5 minutes. Then, the temperature increases at 2.5ºC/min until the final temperature of 220ºC which was also maintained for 5 more minutes (total required time for one sample was 1 hour). In those conditions, LP226A1 has showed a retention time of 37.8 minutes.

6. Affinity chromatography using LP226A1-bound nitrocellulose membrane

6.1. Material and reagents

-16 pieces of 1cm^2 nitrocellulose membrane

-**Casein solution:** 5% (w/v) non-fat dry milk in TTBS

-**Drug solution:** 20mg/ml (60mM) LP226A1 in TTBS

-TTBS: 0.1% (w/v) tween20, 140mM NaCl, 20mM Tris-HCl pH 7.5

-Sample: 2mg/ml soluble protein (see section ***1.1***) in 140mM NaCl, 50mM NaCl pH 7.5 (with or without 0.1%(v/v) tween-20).

-Wash buffer: 0.1% (p/v) tween20, 140mM NaCl, 1mM EDTA, 1mM EGTA, 20mM Tris-HCl pH 7.5 (with or without 0.1%(v/v) tween-20)

-Elution buffer: 2% SDS, 140mM NaCl, 1mM EDTA, 1mM EGTA, 20mM Tris-HCl pH 7.5

-**Loading buffer for western-blot 1X:** 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, 5% (v/v) 2-mercaptoetanol, 1% (w/v) SDS, 20mM Tris-HCl pH 6.8

6.2. Method principle (see section 5.1.2)

The presence of LP226BPs at the soluble protein fraction (see section ***1.1***) was tested by affinity chromatography using nitrocellulose as a stationary phase.

In the present work, casein is used to block the nitrocellulose membrane, it facilitates specific and avoid non-specific binding.

6.3. Procedure

a) Sixteen 1cm^2 nitrocellulose membranes were incubated with or without the drug solution for 30 minutes.
b) Membranes were washed three times with TTBS.
c) When membranes were dried, they were incubated with or without casein for 30 minutes at room temperature. After that, they were washed twice gently transferring each one at an Eppendorf with 1 ml of TTBS.
d) Membranes were incubated with or without the soluble protein and with or without 0.1%(v/v) tween 20 overnight at 4ºC.
e) Membranes were washed twice gently with wash buffer with or without tween. ***(Fig. 3)***

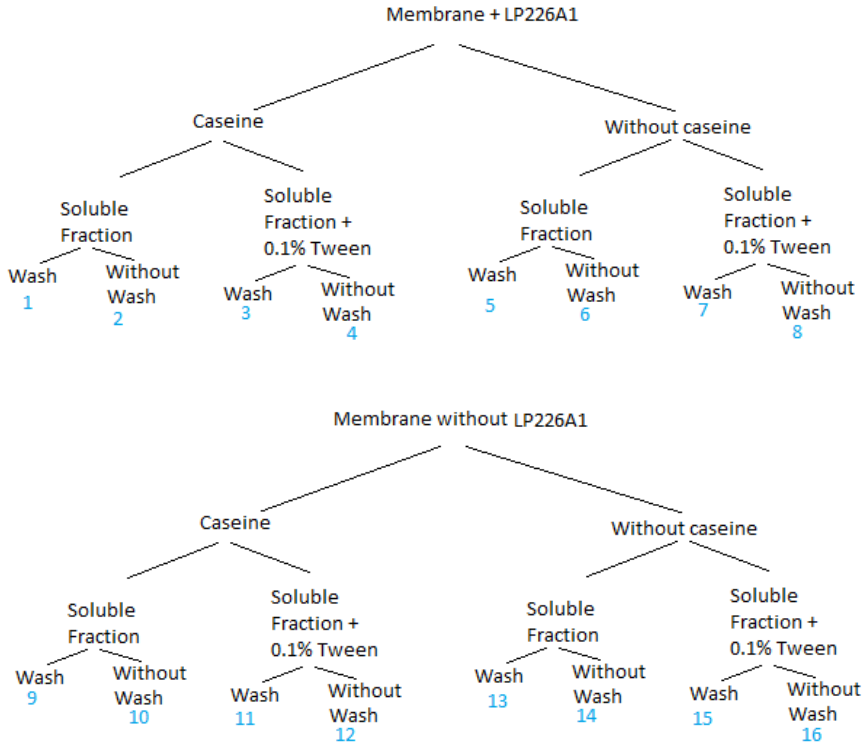

Fig. 3. Treatments applied at the different nitrocellulose membranes. *On the top are located the membranes that were incubated with the drug and at the bottom there are the membranes that were incubated without the drug. The same conditions were applied at the membranes although they were incubated with or without LP226A1. Membranes were incubated with or without casein and with the soluble fraction in tween presence or without it. After that incubation some membranes were immediately dried and some of them were washed with the respective buffer.*

f) The membranes were incubated with 100µl of elution buffer. Twenty µl of the resulting solution from each piece of nitrocellulose were mixed with 5µl of loading buffer for western blot.

g) Finally, the samples were electrophoresed in 14% poly-acrylamide gels and stained with Coomassie Blue (See section ***7*** and ***8***).

7. SDS-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

7.1. Material and reagents

-9.5% acrylamide running gels: 9.5% (w/v) acrylamide, 0.25% (w/v) bis-acrylamide, 0.1% (w/v) SDS, 0.06 (w/v) ammonium persulfate, 0.12% (w/v) TEMED, 150mM Tris-HCl pH 8.8)

-4% acrylamide stacking gels: 4% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 0.1% (w/v) SDS, 0.06% (w/v) ammonium persulfate, 0.12% (w/v) TEMED, 150mM Tris-HCl pH 6.8.

-Electrophoresis buffer: 200mM Glycine, 0.1% (w/v) SDS, 50mM Tris-HCl pH 8.3

-Mini-protean II Electrophoresis system (Bio-rad)

7.2. Method principle

Electrophoresis is a technique that allow a spatial separation of proteins in acrylamide gels according to their molecular weight under the influence of a uniform electric field. Specifically, the smaller molecules migrate more easily than larger ones. The gels are mounted in a chamber with electrophoresis buffer which allow the flow of the electrical field.

Proteins are separated in presence of the anion detergent sodium dodecyl sulphate (SDS). The polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. (Sambrook J.; Fritsh E.F.; Maniatis T.;, 1989)

Using markers of known molecular weight, it is possible to estimate the molecular weight of the polypeptide chains.

In this case, SDS-polyacrylamide gel is carried out with a discontinuous buffer system in which the buffer in the reservoirs is different of the buffer used to the gel. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone on the surface of the running gel. This concentrate all the proteins of the sample into a very small volume which increases the resolution of the gels. Specifically, the sample and the stacking gel contain Tris-HCl at pH 6.8, the electrophoresis buffer have an 8.3 pH and the running gels have an 8.8 pH.

The range of separation of the gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross-linking.

7.3. Procedure

a) All the reagents for the running gel were mixed and applied to a glass chamber where the space between glasses coincide with the desired thickness for the gel. Also, a thin layer of isopropanol was added to avoid the bubble formation.
b) Once the gel was polymerised, the isopropanol was removed and the gel surface was washed with water.
c) Reagents for the stacking gel were mixed and applied on the running gel. Before polymerisation of that gel, the comb was inserted to generate wells for the sample loading.
d) After the stacking gel polymerisation, the comb was removed and the wells washed with water.
e) Gels were placed into the electrophoresis system with the electrophoresis buffer and the samples were loaded into the wells.
f) To run the gel, an electric field was applied at a constant amperage: 20mA per gel.
g) Electrophoresis was finished when the blue front left from down part of the gel. Thus, the gel was removed from the glass chamber.

8. Coomassie Blue Staining

8.1. Material and Reagents

-Coomassie Blue Solution: 0.25 % (w/v) Coomassie Brilliant Blue, 10% (v/v) acetic acid, 50% (v/v) methanol.

-Wash solution: 5% (v/v) methanol, 7.5% (v/v) acetic acid

8.2. Method principle

Proteins separated with electrophoresis can be stained with Coomassie Blue. Coomassie Brilliant Blue binds electrophoresed proteins allowing visualization of the proteins as blue bands within the translucent matrix of the gel.

8.3. Procedure

-The electrophoresed gels were incubated overnight at room temperature or 120 minutes at 60ºC with the coomassie blue solution in a horizontal shaker.

-Gels were washed up with wash solution. Wash solution was changed every two-three hours.

9. Western Blot (WB)

9.1. Material and Reagents

-Sample cocktails containing 2mg/ml of protein in loading buffer for western blot. Samples from human origin were prepared for WB *(Section **1.2**)*.

Table 1.

Patient	Braak Stage	Gender	Age	PM delay	Patient	Braak Stage	Gender	Age	PM Delay
A11/024	NNL	Female	54	06h 45m	**A14/029**	Braak II/A	Female	78	03h 15m
A11/046	NNL	Female	52	05h 45m	**A10/102**	Braak II/B	Male	80	03h 30m
A13/077	NNL	Male	59	08h 30m	**A11/104**	Braak II/B	Female	72	08h 30m
A14/003	NNL	Female	51	04h	**A09/112**	Braak III/0	Male	73	04h 15m
A14/023	NNL	Female	54	08h	**A09/021**	Braak III/A	Male	77	11h 15m
A14/030	NNL	Male	54	08h 45m	**A09/124**	Braak III/A	Male	77	05h 40m
A10/098	Braak I/0	Female	73	15h 45m	**A10/080**	Braak III/A	Female	82	03h 05m
A11/102	Braak I/0	Male	66	12h 10m	**A12/047**	Braak III/A	Male	81	05h 05m
A11/075	Braak I	Male	61	04h 30m	**A09/030**	Braak III/B	Male	86	03h 50m
A11/084	Braak I/A	Male	77	06h 55m	**A09/074**	Braak III/B	Male	82	12h 30m
A11/055	Braak II/0	Male	60	04h 15m	**A10/013**	Braak III/B	Female	76	03h 50m
A12/009	Braak II/0	Male	78	14h 14m	**A10/086**	Braak III/B	Male	76	06h
A12/086	Braak II/A	Male	75	05h 15m	**A09/111**	Braak III/C	Male	77	13h 45m
A13/070	Braak II/A	Female	79	10h	**A12/038**	Braak IV/B	Male	79	04h 15m

List of used samples for western blot. That samples proceeded from human hippocampus brain patients classified by Braak Stage, gender, age and post mortem delay (PM delay).

-**9.5% (w/v) polyacrylamide gels**

-Electrophoresis buffer: 200mM Glycine, 0.1% (w/v) SDS, 50mM TrisH-Cl pH 8.3

-Transference buffer: 200mM Glycine, 20% (v/v) methanol, 50mM Tris-HCl pH 8.3

-Nitrocellulose membranes (GE, Amersharm)

-Blocking solution: 5% (w/v) non-fat dry milk and 1% (w/v) BSA in TTBS
-Primary antibody solution: 1/1000 antibody Anti FA2H (Fatty Acid 2-hydroxylase; Proteintech) with 2.5% (w/v) milk and 0.5 (w/v) BSA in TTBS
-**Secondary Antibody (Anti-rabbit):** 1/5000 antibody Anti-rabbit (GE Healthcare) in TTBS
-**Odyssey Infrared Imaging System** (LI-COR Inc.)

9.2. Method principle

Samples for this assay were prepared following the protocol showed in section ***1.2***. Those samples proceed from AD's patient brains and patients with non-neuropathological lesions as well.

Western blot was used to detect fatty acid 2 hydroxylase (FA2H) protein (43KDa). It consists in an electrophoresis to separate sample protein according to its molecular weight (following ***7.3*** procedure), a transference of proteins from the gel to a nitrocellulose membrane and a posterior incubation of this membrane with the primary and secondary antibodies which allow specific detection and quantification. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The optical density of the band correlates the amount of protein present. (Mahmood & Yang, 2012)

9.3. Procedure

-Human hippocampus protein samples were separated by SDS-PAGE method (following procedure in section ***7.3***) in 9.5% (w/v) polyacrylamide gels.
-Proteins were transferred to nitrocellulose membranes in transference buffer at 350mA for 2 hours at 4ºC.
-When protein were transferred, the membrane was blocked with the blocking solution at room temperature for one hour. This solution blocks all the empty spaces at the membrane and avoids the non-specific antibody binding to nitrocellulose.
-The membranes were then probed overnight at 4ºC with the primary antibody solution and washed three times with TTBS 5 minutes in a horizontal shaker at room temperature to eliminate the unbound antibody.
-The primary antibody is detected via a fluorescent-labelled secondary antibody (incubation for 1 hour at room temperature).
-The unbound secondary antibody was washed three times for 5 minutes each with TTBS and once with TBS (without tween-20) in a horizontal shaker at room temperature.
-The fluorescence coming from the specific protein was detected using the Odyssey Infrared Imaging System and quantified by densitometry via Image J software. Optical density from FA2H protein was normalized with α-tubulin levels.

RESULTS AND DISCUSSION

1. IEX Chromatography and radio-binding assay results

Figure ***Fig. 4*** shows a representative IEX chromatogram. We have plotted the percentage of elution buffer (containing 1M NaCl) mixed with starting buffer in order to generate a discontinuous stepwise gradient with different salt concentrations. Conductivity (mS/cm) and absorbance at 280nm (ultraviolet:UV) (absorbance units: mAU) are also showed. The conductivity is associated with NaCl concentration. In each step of the discontinuous gradient ***(Fig. 4)*** the system changes the mixture of elution buffer and starting buffer to elevate ionic strength of the medium and induce protein elution from the column. Therefore, each increase in NaCl concentration is followed by conductivity elevation and protein elution as showed by UV signal.

In this experiment, proteins elute from the column when conductivity increases excepting for the first UV peak obtained which is eluted whereas NaCl concentration is still the same than in the starting buffer (0mM NaCl) (see ***Fig. 4***). This peak matches with the protein sample that does not bind to the column and elute as is. The following peaks match with those that eluted from the column when NaCl concentration was increased up to 100, 200, 300, 400, 500 and 1M. The protein fractions eluted with 200mM and 500mM NaCl did not show a single peak. In those cases, the protein fraction eluted in two phases, probably because they eluted at different salt concentrations that succeed into the same step of the salt gradient. Finally, the fraction eluted with 1M NaCl corresponds to the protein sample that was retained more strongly (highest net negative charge). Each salt elution step is applied with 5 column volumes and they are collected into 5 fractions (1 ml each). However, only the three central fractions (fractions 2, 3 and 4) of the same gradient step contain the eluted protein peak.

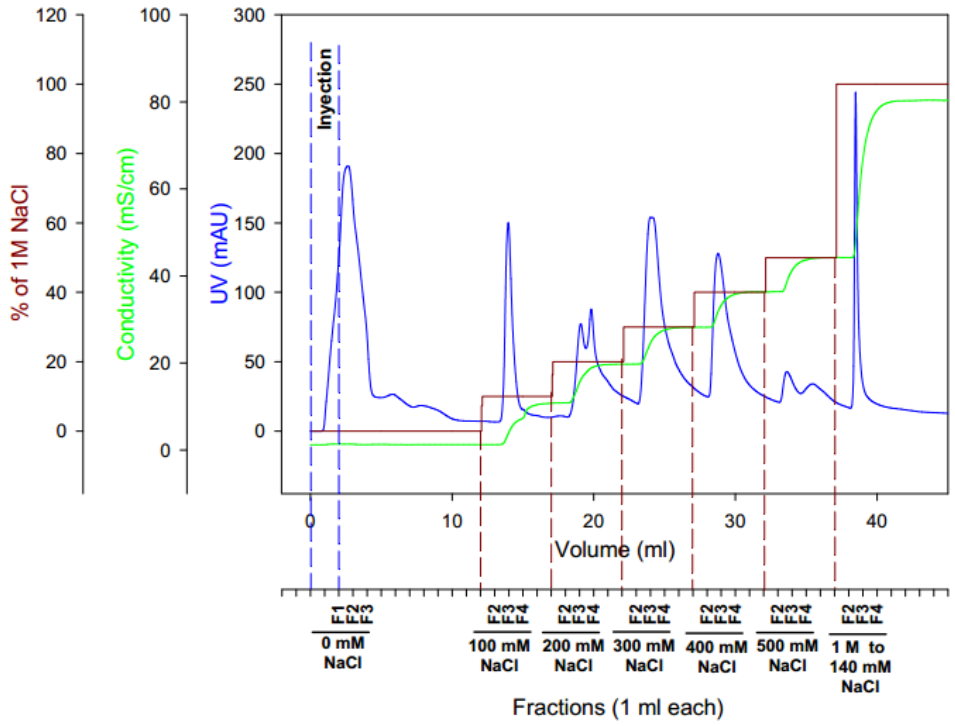

Fig. 4. Chromatogram obtained by IEX with Resource Q column*. The percentage of elution buffer (containing 1M NaCl) applied to the column is shown by a red line; conductivity (mS/cm) is shown by a green line, and finally, absorbance at 280nm (UV in mAU) is shown by a blue line.*

The same peak pattern was observed after repeating the experiment three times. The only difference found was that, when the starting protein concentration varies, the intensity of the UV baseline peaks obtained also fluctuates. Indeed, UV signal intensity of the observed peaks highly correlates with the initial sample concentration demonstrating that the experiment has a high reproducibility.

The aforementioned protein fractions obtained from IEX chromatography were subjected to radio binding assay. Specifically, only fractions 2, 3 and 4 corresponding to the protein peak eluted with each gradient step were subjected to this assay (see experimental procedures in section ***4.3).*** Data from scintillation counter is shown as counts or disintegrations per minute (cpm or dpm). The difference between the counts per minute from the total binding reaction and the non-specific binding reaction belongs to the signal from specifically bound ^{3}H-LP226A1. Following the unit conversion: $2.22.10^{12}$ cpm are 1 Ci and taking into account the specific activity of our ligand (0.6nCi/pmol), the pmols of ^{3}H-LP226A1 specifically bound to sample proteins were calculated *(**Fig. 5**).*

Figure ***5*** shows ^{3}H-LP226A1-binding results for all the fractions analysed (the average of 3 different experiments). Two peaks showing more than 10 pmol of bound ^{3}H-LP226A1 per mg of protein were observed. Those peaks were eluted when concentrations of 300mM and 400mM NaCl were applied to the column; meaning that LP226BPs were present in such fractions. In addition, a lower-intensity peak was appreciated when elution was done with 100mM NaCl concentration.

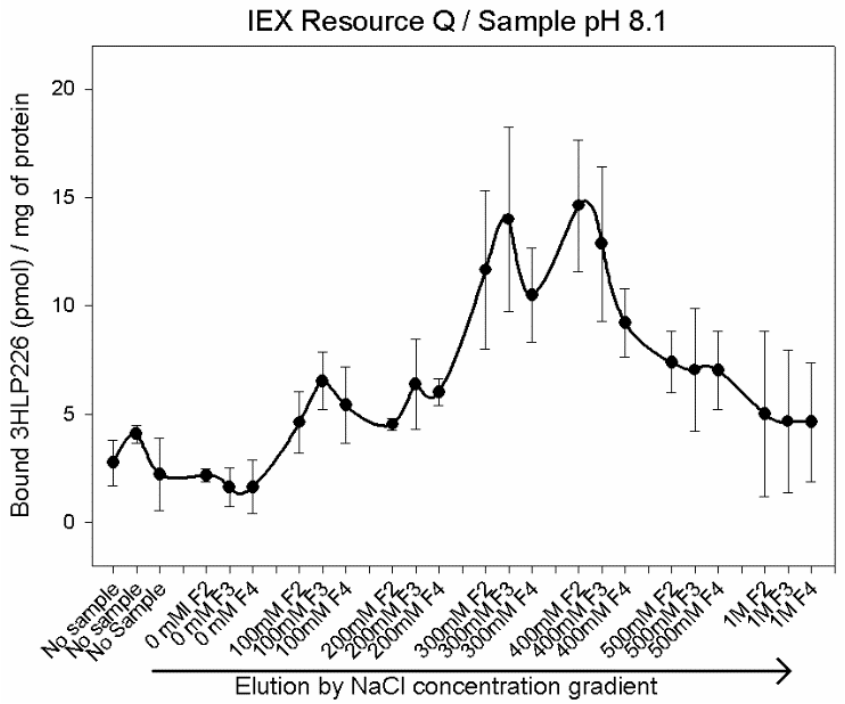

Fig. 5. Specific binding of ^{3}H-LP226 to protein samples from IEX chromatographic fractions. *Binding analysis comprised 3 controls without any protein (blanks), 3 fractions containing column unbounded proteins (0 mM NaCl) and the 3 central fractions (2, 3 and 4) obtained from each gradient step (elution with 100mM, 200mM, 300mM, 400mM, 500mM and 1M of NaCl). The peaks that indicate the interaction between ^{3}H-LP226A1 and LP226BPs are mainly those that eluted with 300 and 400mM NaCl.*

Central fractions (2, 3 and 4) from each step of the discontinuous NaCl gradient were subjected to electrophoresis and coomassie blue staining to verify that stepwise NaCl gradient correlates with different protein elution. As shown in ***Fig. 6***. the different proteins samples were separated according their molecular weight by SDS-PAGE. For each salt step, a different band pattern was obtained so that different proteins were eluted in each gradient step. As expected, the results also shown that fraction 3 (central fraction) of each gradient step contained the highest protein concentration. These data correlates with the presence of the UV peak in this fraction (see ***Fig. 4***). On the other hand, even though the column unbound sample (elution with 0mM NaCl) contained proteins (see ***Fig. 4*** and ***Fig. 6***) they showed no binding to ^{3}H-LP226A1 (see ***Fig. 5***). In this sense, no ^{3}H-LP226A1 binding was either found for protein fractions that eluted with 1M NaCl. However, just in this case, the presence of protein was quite low (see ***Fig. 4*** and ***Fig. 6***), suggesting that almost all proteins eluted from the column before applying the highest NaCl concentration.

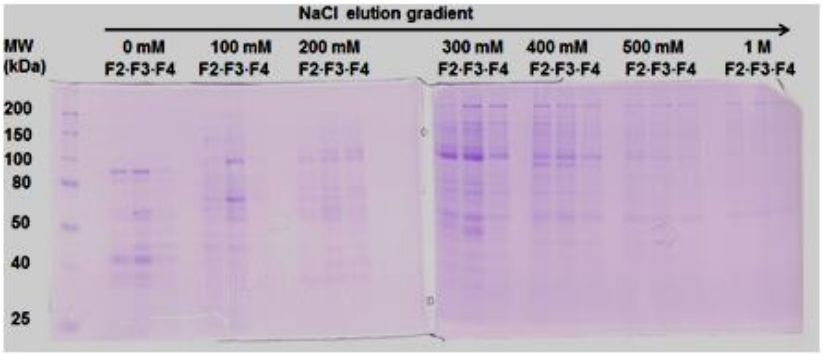

Fig. 6. Coomassie blue stained gels obtained after electrophoresis of IEX fractioned proteins by NaCl discontinuous gradient. *On the first left lane, it was loaded molecular weight markers could be seen with their respective molecular weight (KDa) whereas followed lanes where loaded with the three fractions (2, 3 and 4) obtained through stepwise gradient elution (0, 0.1, 0.2, 0.3, 0.4, 0.5 and 1M NaCl) and previously tested by radio-binding assay.*

2. HIC and Radio-binding assay results

Fractions 2, 3 and 4 from IEX elution with 300mM NaCl (see ***Fig. 5***) were used as sample to perform additional protein separation by HIC. The resultant chromatogram is shown in ***Fig. 7***.

Protein sample from IEX were adjusted to contain a final concentration of 2.5M NaCl and 0.5% CHAPS (see sample preparation in section ***1.1.3***). Sample injection was followed by the first UV peak containing the major part of the sample (see **Fig. 7**). This protein peak belongs to the unbound sample. Subsequently, as NaCl concentration was going down, conductivity levels decreased causing additional protein elution from the column (see **Fig. 7**). This elution was performed via NaCl stepwise gradient elution, being NaCl starting concentration (sample concentration) 2.6M. Subsequent gradient steps were 2, 1.5, 1, 0.5 and 0.14M NaCl (see method principle in section ***3.2***). After stepwise elution, a final step with a buffer that containing 0.14M and 1% (w/v) CHAPS was applied to elute remaining proteins from the column.

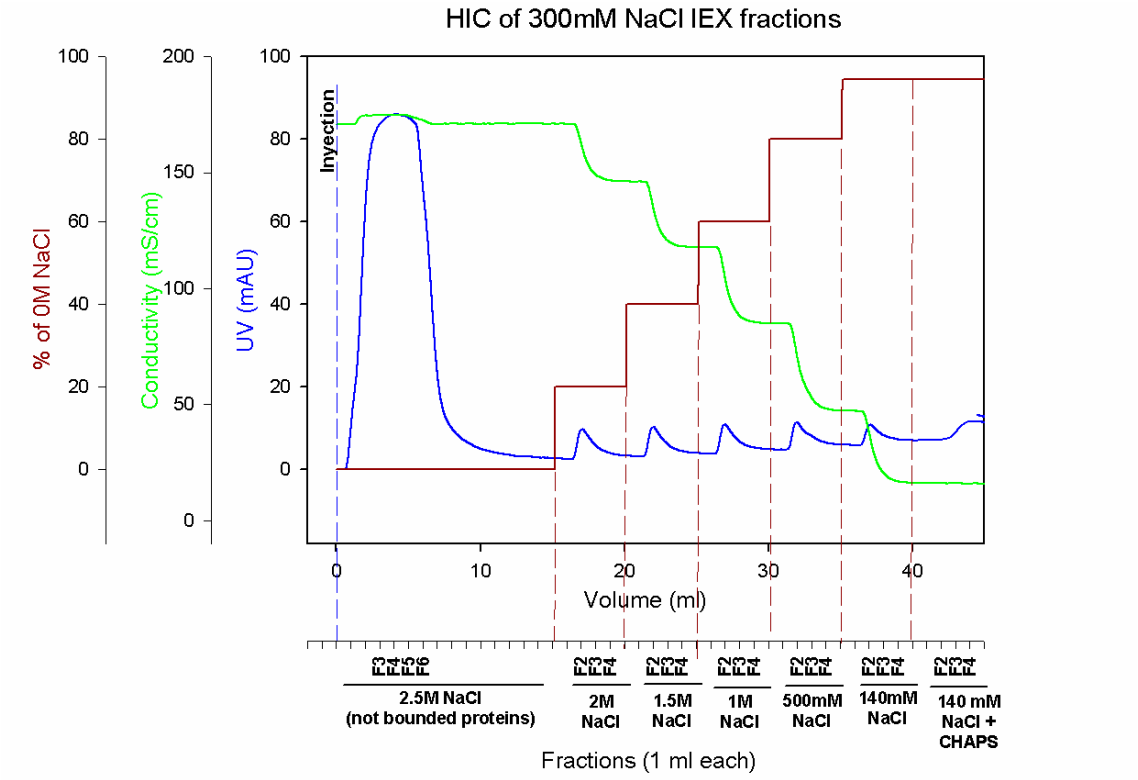

Fig. 7. Chromatogram obtained by HIC assay. *Percentage of elution buffer (without salt, 0M NaCl) that was applied to the column by stepwise gradient elution is shown by a red line. Eluted protein from the column is monitored via UV (mAU) absorbance (blue lane). First eluted protein peak belongs to unbound sample whereas following peaks belongs to proteins peaks eluted with 2M, 1.5M, 1M, 500mM and 140mMNaCl. Conductivity (mS/cm) is plotted as a green lane.*

The first eluted UV peak (unbound sample) contained the main part of the injected protein so that it may be concluded that the major part of the sample did not bind the column hydrophobic matrix under current experimental conditions. However, when the percentage of elution buffer (without NaCl) increased and the conductivity decreased, minor UV peaks appeared, indicating that part of the initial protein was retained and this eluted after each salt gradient step. The eluted protein (UV peak) was mainly located in fractions 2, 3 and 4 of each salt gradient step. For this reason, radio-binding assay was performed with these fractions as we did previously with IEX fractions (see ***Fig. 5***). Obtained results are showed in ***Fig. 8.***

First, two controls that did not contain protein sample (blank) were analysed by radio-binding assay to ^{3}H-LP226A1. Those reactions did not show H^{3}-LP226A1 binding (see ***Fig. 8***). Fractions 3, 4, 5 and 6 matching the column unbounded sample displayed evident ^{3}H-LP226A1 binding indicating presence of LP226BPs in these fractions. Nevertheless, not all LP226BPs were concentrated at the unbounded sample since those protein fractions which eluted with eluted with 2M and 1M NaCl also shown binding peaks (see ***Fig. 8***). The protein fraction eluted with 2M NaCl shown a peak of 7.29pmol of ^{3}H-LP226A1/mg of protein and the fraction eluted with 1M NaCl shown a peak of 5.01pmol/mg of protein. The fraction eluted with 1.5M NaCl shown just minor ^{3}H-LP226A1 binding beak. Finally, binding reaction with those fractions that eluted with 500mM and 140mM of NaCl displayed no binding to ^{3}H-LP226A1, indicating no presence of LP226BPs at these samples.

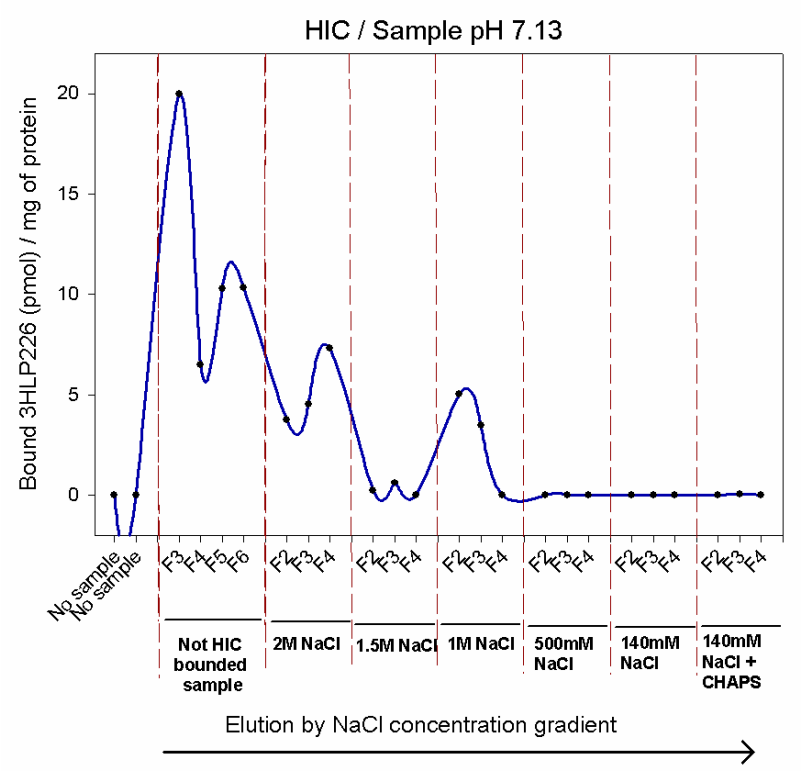

Fig. 8. Specific ^{3}H-LP226A1 binding to protein fractions from HIC. *Two control samples without protein were used as blanks. Four fractions from the column unbound sample and 3 fractions corresponding to proteins eluted by NaCl stepwise gradient: 2M, 1.5M, 1M, 0.5M and 140mM of NaCl. The peaks indicating the interaction between ^{3}H-LP226A1 and proteins of the sample are present at the unbounded sample and in the fractions eluted with 2M and 1M NaCl.*

It is remarkable that major protein binding to ^{3}H-LP226A1 was found in protein samples belonging to the column unbound fractions. Consequently, this result suggests that binding peaks eluting with 2M and 1M NaCl may contain LP226BPs that were also present at the unbound samples. It means that certain LP226BPs would be retained only partially with one part of the protein eluting as it (unbound protein) and one part of it eluting after NaCl concentration down-regulation. Nevertheless, LP226BP chromatographic fractionation (even if this fractionation may be partial) is the principal aim of this experiment because those fractions that eluted with 2M and 1M NaCl contains semi-purified LP226BP which may be subjected to additional chromatographic separation to get complete LP226BP purification. In this sense, it is noteworthy that HIC assays are normally performed by using sulphate salts to increase the hydrophobic character of the target protein instead of NaCl or KCl. Just in this case, sulphate salts were avoided because previous results demonstrated incompatibility between the detergent CHAPS and sulphate salts such us ammonium sulphate or sodium sulphate. In fact, even with low concentrations of CHAPS, addition of ammonium or sodium sulphate induced detergent precipitation making unviable HIC using these compounds with our original sample. Therefore, further research in this sense would be necessary in order to establish better HIC experimental conditions.

As we did before for IEX fractions (see ***Fig. 6***), HIC chromatographic fractions were also subjected to electrophoresis by SDS-PAGE and posterior coomassie blue staining (***Fig. 9***). As expected, our results showed a few protein bands in fractions belonging to column unbound proteins. Unsurprisingly, the observed band pattern was quite similar to that previously shown in ***Fig. 6***

for IEX protein fraction that eluted with 300 mM NaCl. However, no stained protein was observed in fractions from the NaCl stepwise gradient. Since protein presence was demonstrated by UV peaks as well as ^{3}H-LP226A1 binding peaks in protein fraction eluted with 2M and 1M NaCl, it is reasonable to hypothesize that protein concentration in such fractions is too low to be detected by coomassie brilliant blue (sensibility around 30 ng of protein (Bio-Rad, 2012)). Protein staining for HIC samples will be addressed by silver staining in future experiments (sensibility range is 50-100 more sensitive than colloidal Coomassie Blue staining (Simpson, 2007)).

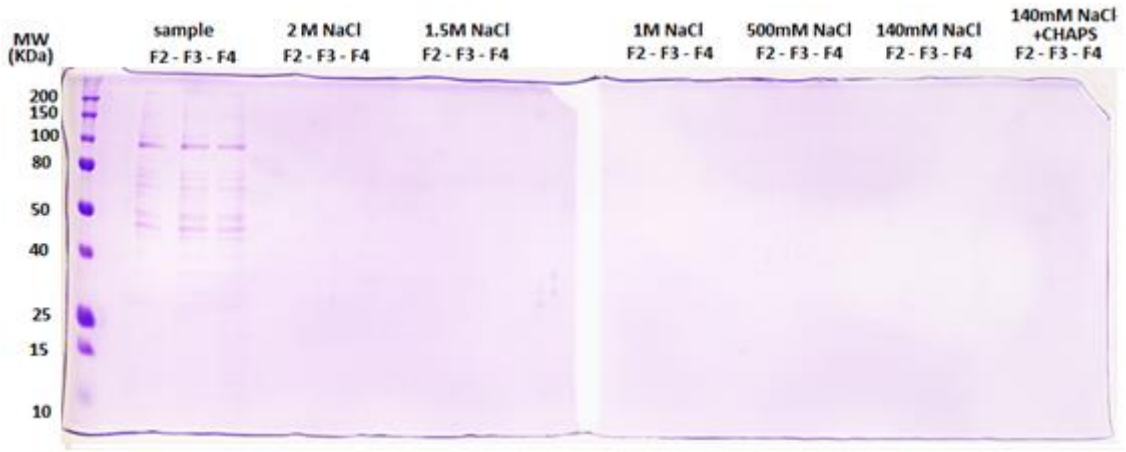

Fig. 9. Coomassie blue stained gels obtained after the electrophoresis of the HIC fractioned proteins applying a NaCl discontinuous gradient. *On the first left lane, it was loaded molecular weight markers could be seen with their respective molecular weight (KDa) whereas followed lanes where loaded with the three fractions (2, 3 and 4) obtained though stepwise gradient elution (2, 1.5, 1, 0.5, 0.14 M NaCl and with 0.14M NaCl and 1%CHAPS) and previously tested by radio-binding assay. .*

3. Affinity chromatography

Firstly, different conditions were tested to favour LP226A1 binding to nitrocellulose membrane in order to use LP226A1-bound nitrocellulose as stationary phase for affinity chromatography. As mentioned previously, LP226A1 showed a retention time of 37.8 minutes when the sample was analysed by gas chromatography using the Supelco SP-2330 column (see section ***5.2.3***). The presence or absence of LP226A1 on the membrane was tested by analysing this peak intensity. Our results demonstrated that LP226A1 incubation in TTBS was the best condition to promote LP226A1 binding to nitrocellulose (***Fig. 10***).

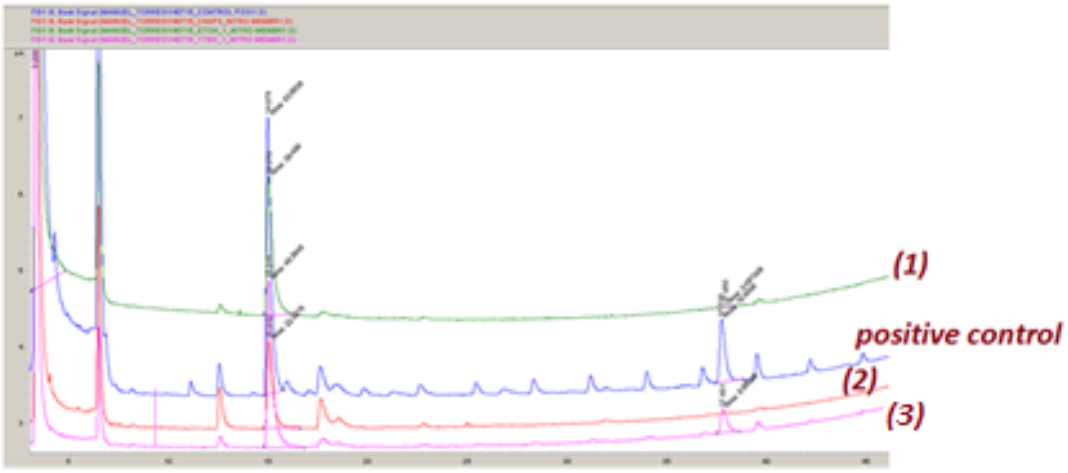

Fig. 10. Chromatogram obtained with GC showing presence or absence of LP226A1 at retention time of 37.8 min*. Samples 1, 2, 3 and one positive control are represented. Neither sample 1 (membrane incubated with LP226A1 in 5% ethanol) nor sample 2 (membrane incubated with LP226A1 in 1% Chaps) showed LP226A1 peak at 37.8 minutes indicating that LP226A1 was not bind to nitrocellulose under these conditions. In contrast, Sample 3 (membrane incubation with LP226A1 with TTBS) shown a peak at 37.8min indicating presence of LP226A1.*

As showed in ***Fig. 10,*** not all conditions promote LP226A1 binding to nitrocellulose. Nitrocellulose membrane pieces which were incubated in presence of 5% (v/v) ethanol or in 1% (w/v) CHAPS were dissolved in methanol prior to lipid extraction and derivatization. None of these conditions favoured LP226A1 binding to nitrocellulose (see figure 10). These data indicate that ethanol and CHAPS did not allow LP226A1 binding to nitrocellulose membrane. However, when nitrocellulose membrane piece was incubated with LP226A1 in TTBS, a peak at 37.8 minutes was clearly observed. This indicates that, in presence of TTBS, the drug bound to nitrocellulose and posterior washes with the same buffer (TTBS) were not able to get rid the drug from the membrane so that LP226A1 anchorage to nitrocellulose was irreversible under these experimental conditions. Peak intensity revealed that the amount of LP226A1 bound to nitrocellulose was 63.1µg/cm^2. Taking into consideration that membrane capacity is 80µg/cm^2 (GE Healthcare) in terms of protein (Bio-Rad, 2012), this results indicates that nitrocellulose membrane is almost saturated with LP226A1 when incubation is performed with TTBS.

Once the best condition to promote binding of LP226A1 to nitrocellulose was determined, different conditions were tested to check if nitrocellulose-anchored LP226A1 interact with LP226BP from the soluble fraction of proteins. No membrane proteins were assayed by this method because our previous results showed that CHAPS detergent extract LP226A1 from nitrocellulose making unviable this technique with such sample (see ***Fig. 10***). In order to test the best condition to promote LP226A1 interaction with soluble potential receptors, the experiment was performed with or without casein blocking or incubating soluble protein fraction in presence or absence of tween-20. Membrane bound proteins were eluted with 2% (w/v) SDS, electrophoresed and stained with coomassie blue. Our results showed the same (or very similar) protein band pattern when comparing those membranes incubated with or without suggesting that detected proteins were bound to the nitrocellulose membrane in a non-specific manner instead of to the LP226A1 ligand anchored to nitrocellulose ***(Fig. 11)***.

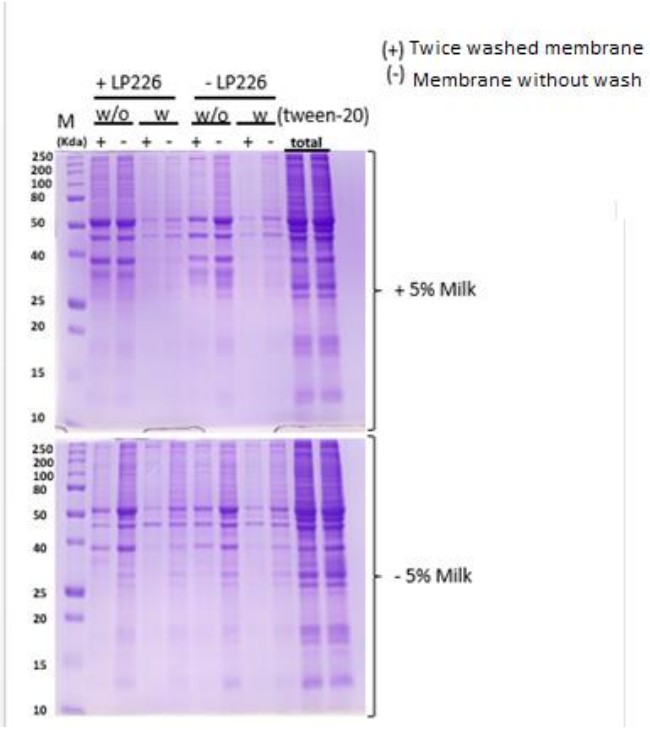

Fig. 11. Coomassie blue stained gels of the samples obtained incubating the membrane in different conditions. *At the top, the gel blocked with 5% casein (milk) and at the bottom there is the gel with de samples obtained not-blocking the nitrocellulose membrane with 5% (w/v) milk. In both gels in the first lane the molecular weight marker can be seen; it is followed by four lanes of the samples that were incubated with the drug without tween-20 (with and without washes) and with tween-20 (with or without washes). In the four posterior lanes there are the samples obtained without incubating the nitrocellulose membrane with the LP226A1 but treated in the same way that the incubated membranes. Finally, the two last lanes have the total soluble fraction protein sample.*

In addition, both gels (nitrocellulose membrane pieces blocked with or without 5% (w/v) milk) nearby had no differences. This result demonstrate that the incubation of nitrocellulose pieces with this solution did not affect at the membrane protein union (***Fig. 11***).

On the other hand, the protein band pattern for samples incubated with or without tween-20 is different. This demonstrates that the presence of tween in the sample reduces protein binding to nitrocellulose (in presence of tween-20 a lower amount of protein was bound the membrane) but do not affect interaction of LP226A1 with LP226BPs which was not detected (with or without tween-20).

In the present work we have considered nitrocellulose membrane as a support to create an affinity chromatography instead of covalently link LP226A1 to a certain resin. This last option was avoided because a chemical reaction to link LP226A1 to a certain functional group normally implies reaction among hydrophilic radicals of molecules. In the case of LP226A1, the unique hydrophilic group is the fatty acid polar head (carboxyl group). In addition, we currently hypothesize that LP226A1 α-hydroxyl group is a key structure implicated in the neuroprotective role of this molecule. Since the LP226A1 α-hydroxyl group is closed to the carboxyl polar head, linkage of the molecule via carboxyl group would impede geometrical access of other molecules to the hydroxyl group of LP226A1. That is because this methodology was discarded and alternative ways like using nitrocellulose as support were addressed.

4. Western Blot analysis

In this analysis, the protein expression of FA2H was analysed in hippocampus to compare the different Braak stages of Alzheimer's patients among them. To compare results from different experiments, three NNL (no neuropathological lesion controls) were used as internal controls and systematically loaded in all our experiments. As shown in ***Fig. 12*** several non-specific bands appeared and one specific band at 43KDa was detected in human and mouse brain samples. Since this band was more clearly detected in mouse samples, this sample was used as a positive control and loaded at the first lane for each experiment.

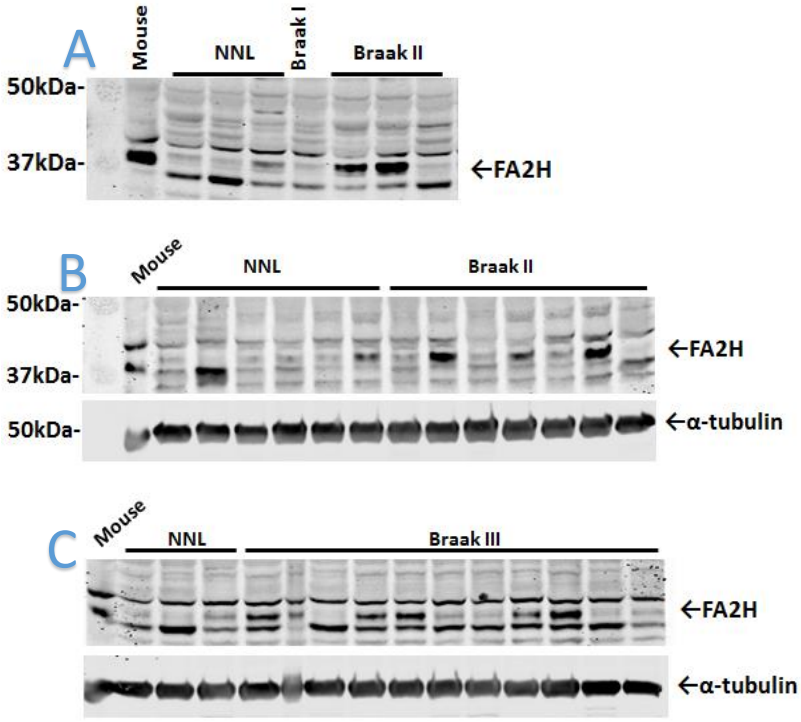

Fig. 12. Western blot analysis of FA2H protein expression in hippocampus*. In each membrane, firstly a mouse sample was loaded as a positive control followed by NNL (no neuropathological lesion) controls and AD patients samples (Braak I, II or III). The band corresponding at the FA2H protein can be detected between 50kDa and 37KDa markers bands because it molecular weight is 43KDa* (Alderson et al., 2004).

Quantitative analysis is showed in ***Fig. 13.*** The 100% value was allocated at the patients with non-neuropathological lesions because they are the control samples. Our results showed that the highest values of FA2H were observed in Braak II samples followed by Braak III samples. These increments in both groups were quite heterogeneous though statistically significant as compared with the controls. Braak I samples showed similar or slightly lower values than NNL samples.

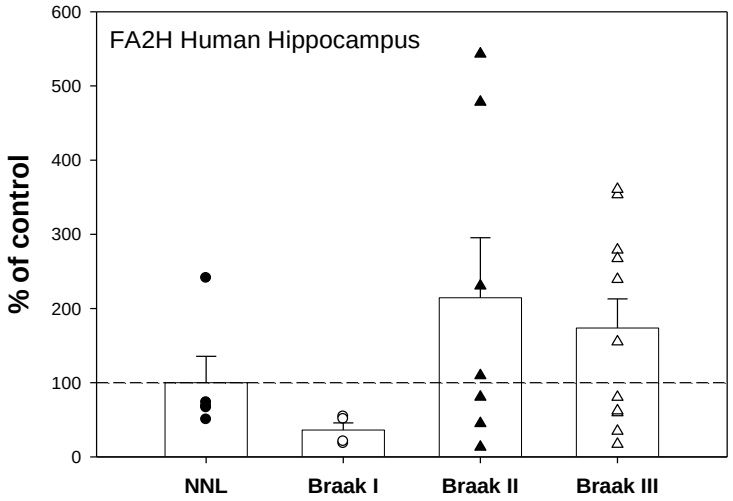

Fig. 13. Human hippocampus FA2H expression results detected by western Blot. *The FA2H expression is represented at the different Alzheimer stages (Braak I, Braak II and Braak III) and compared with the 100% control value allocated at non-neuropathological lesions (NNL) patients.*

The differences in FA2H expression are neither associated at the patient age nor post mortem patient delay. In previous studies, low levels of FA2H have been associated with neuropathology, specifically at brain iron deposition (Pierson et al., 2012). However, in these experiments half of the patient samples analysed in Braak II and Braak III showed increased FA2H levels as compared with NNL control samples. This increment could hypothetically be produced by the brain neurons to counteract the beginning of the neuropathology and protect themselves. In that case, this response would demonstrate an early self-neuroprotective response of human neurons against AD neuropathology.

In this sense, since LP226A1 is an α-hydrosylated hydroxyl fatty acid, the increment of FA2H expression may corroborate neuroproctective role of LP226A1.

Nowadays, AD cannot be diagnosed until a relatively advanced stage (Braak III), but here we have demonstrated that FA2H expression is significantly increased in one pre-clinical stage (Braak II). These results suggests that this enzyme could be used as a biomarker to diagnose AD before first cognitive symptoms appear. Nevertheless, more experiments should be performed extending sample number and stage to confirm several premises: (1) confirm the observed expression increment of FA2H in Braak II and III, (2) check if FA2H expression in Braak I is statistically decreased as compared with controls, (3) check if elevated expression of FA2H remains at advanced AD stages like Braak V and VI.

CONCLUDING REMARKS

In this work, we used different methods to find LP226A1 receptors. We used non-carrier seven month female cortex brains to find possible proteins that bind the drug. IEX and HIC chromatography performed with sodium chloride permitted fractioning membrane proteins. Posterior binding assays showed that the possible receptor(s) eluted with 300mM and 400mM of salt at IEX chromatography and with 1M and 2M of NaCl in HIC chromatography.

Unfortunately, the assays to design the affinity chromatography could not help to concretise that research. Because primarily, the bounded LP226A1 eluted in presence of 1% Chaps indispensable for analysing the membrane fraction and secondly, when we applied the soluble fraction proteins, they did not bind specifically at the drug, they bind the nitrocellulose membrane.

On the other hand, we found that different AD stage patients showed different FA2H expression levels. Concretely, Braak II stage patients present the highest levels being followed by Braak III stage patients; those results could be indicative of an alleged auto-neuro-protective respond of the brain that wanted to compensate the symptoms of the disease. These results could potentially help to understand why the 2-hydroxylated DHA could act as a neuroprotector.

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