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EVALUATION OF NATURAL COMPOUNDS ACTIVITY AGAINST OXIDATIVE STRESS AND NEUROINFLAMMATION. IMPLICATIONS FOR ALZHEIMER'S DISEASE

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Evaluation of natural compounds activity against oxidative stress and neuroinflammation. Implications for Alzheimer's disease

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Evaluation of natural compounds activity against oxidative stress and neuroinflammation. Implications for Alzheimer's disease

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En Lugo, 20 de septiembre de 2019

Fdo Luis M. Botana López

Fdo Eva Alonso López

A mi bisabuelo,
que puso los cimientos
de esta Universidad

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ABBREVIATIONS AND ACRONYMS

AChE: Acetylcholinesterase
AD: Alzheimer's disease
AMPK: AMP-activated protein kinase
ANT: Adenine nucleotide translocase
APP: Amyloid precursor protein
ARE: Antioxidant response element
Arg-1: Arginase-1
ATP: Adenosine triphosphate
Aβ: Amyloid beta
BBB: Blood brain barrier
C99: C-terminal fragment
CAT: Catalase
Cdk5: Cyclin-dependent protein kinase-5
CK1: Casein kinase 1
CNS: Central nervous system
CypA: Cyclophilin A
CypD: Cyclophilin D
eNOS: Endothelial nitric oxide synthase
ERK: Extracellular signal-regulated kinase
ETC: Electronic transport chain
FDA: Food and Drug Administration
GM-CSF: Granulocyte-macrophage stimulating factor
GPX: Glutathione peroxidase
GR: Glutathione reductase
GSH: Glutathione
GSK3β: Glycogen synthase-3-beta
GSSG: Glutathione disulfide
IC_{50}: Half maximal inhibitory concentration
IKK: IkB kinase
IL-10: Interleukin-10
IL-1β: Interleukin 1-beta
IL-2: Interleukin 2
IL-4: Interleukin-4
IL-6: Interleukin 6
iNOS: Inducible nitric oxide synthase
JNK: c-Jun-N-terminal kinase
Keap1: Kelch-like ECH-associated protein1
LC3: Microtubule-associated light chain 3
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MAPT: Microtubule-associated protein tau
MitoQ: Mitoquinone mesylate

mPTP: Mitochondrial permeability transition pore
mTOR: Mammalian target of rapamycin
NADH: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NFkB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NFTs: Neurofibrillary tangles
NMDAR: N-methyl-D-aspartate receptors
nNOS: Neuronal nitric oxide synthase
NOS: Nitric oxide synthase
NOX: Nicotinamide adenine dinucleotide phosphate oxidase
Nrf2: Nuclear factor E2-related factor 2
NRPS: Non-ribosomal peptide synthase
NSAIDs: Non-steroidal anti-inflammatory drugs
PDPKs: Proline-directed protein kinases
PDR: Pharmacophore directed retrosynthesis
PiC: Phosphate carrier
PKA: Protein kinase A
PKS: Polyketide Synthase
PP2A: Protein phosphatase 2A
PPIase: Peptidyl-prolyl isomerase
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
sAPPβ: Soluble APPβ fragment
SOD: Superoxide dismutase
TGF- β: Transforming growth factor beta
TLRs: Toll like receptors
TNF-α: Tumor necrosis –alpha
TREM-2: Triggering receptor expressed on myeloid cells 2
ULK1: Unc-51-like kinase
VDAC: Voltage-dependent anion channel
VitE: Vitamin E
β-secretase: BACE1
ΔΨm: Mitochondrial membrane potential

INDEX

RESUMEN

El aumento en la esperanza de vida ha provocado un incremento en la prevalencia de las patologías neurodegenerativas, siendo la enfermedad de Alzheimer la más común. A día de hoy no existen tratamientos capaces de frenar el avance de esta dolencia, los medicamentos disponibles sólo proporcionan una mejora temporal de los síntomas. Por ello, la búsqueda de moléculas que puedan alterar el progreso de la enfermedad es un gran reto. En los últimos años se ha llevado a cabo una extensa investigación en este sentido, con cientos de ensayos clínicos. Sin embargo, a pesar del gran esfuerzo los resultados han sido decepcionantes. Una de las razones principales a las que se atribuye el fallo en la búsqueda de nuevos medicamentos es la complejidad de la enfermedad, cuyos mecanismos moleculares todavía no están completamente claros. Además, la mayoría de los tratamientos probados hasta ahora estaban centrados en dos dianas, la proteína tau y el péptido beta amiloide (Aβ), y una estrategia múltiple que afecte a varios de los procesos que ocurren en la enfermedad podría ser la solución para frenar su progresión.

Entre los mecanismos moleculares de la enfermedad de Alzheimer, el estrés oxidativo y la neuroinflamación juegan un papel importante. El primero está provocado por el aumento en la liberación de moléculas tóxicas derivadas de la fosforilación oxidativa que tiene lugar con la edad, así como con la disminución en la efectividad de los sistemas antioxidantes endógenos. El gran consumo de oxígeno del cerebro y su limitada capacidad antioxidante en comparación con otros órganos hace que sea especialmente sensible a este desequilibrio, que puede llegar a producir la muerte celular. Con respecto a la neuroinflamación, la microglía, responsable de la respuesta inmune en el cerebro, se encuentra activada de forma crónica y libera de forma descontrolada mediadores inflamatorios. Estas células se activan debido a la presencia de moléculas tóxicas a su alrededor, y contribuyen a crear un círculo vicioso generando citoquinas y otros mediadores que dañan a las neuronas.

En este sentido, los compuestos naturales, que se caracterizan por su capacidad para modular las defensas antioxidantes celulares y la inflamación, presentan un gran potencial farmacológico para el tratamiento de las enfermedades neurodegenerativas. De hecho, los compuestos que se encuentran en plantas y frutas han mostrado resultados prometedores para el tratamiento y prevención de estas dolencias. Por otro lado, el medio marino es un gran reservorio de moléculas con una inmensa diversidad estructural, y por tanto una gran fuente de metabolitos con capacidad terapéutica.

Durante esta tesis doctoral se han evaluado 45 compuestos de origen marino y 15 bebidas funcionales de frutas para hacer una determinación inicial de su potencial para prevenir y tratar la enfermedad de Alzheimer. Para ello se han utilizado modelos celulares de estrés oxidativo y neuroinflamación, así como modelos específicos de marcadores de esta patología. Finalmente, los efectos neuroprotectores de las bebidas se determinaron en un modelo murino de inflamación.

Nota: En el resumen se han conservado las abreviaturas en inglés para dar continuidad al texto

En primer lugar, se presentan los resultados obtenidos con los compuestos marinos, de los cuales 17 han sido aislados de bacterias, cuatro de esponjas, y 24 se han sintetizado usando como guía un compuesto natural de una esponja, la gracilina A.

En cuanto a las bacterias, el desarrollo de nuevas técnicas de cultivo y herramientas genéticas ha permitido un incremento en el número de compuestos aislados a partir de estos pequeños organismos. Entre ellos destacan las cianobacterias, organismos fotosintéticos que pueden vivir prácticamente en todo tipo de ecosistemas. Esta gran capacidad de adaptación está relacionada con su habilidad para producir diversos metabolitos secundarios, la mayoría de los cuales son péptidos con actividad citotóxica. En esta tesis se ha determinado la actividad biológica de 14 compuestos derivados de cianobacterias pertenecientes a dos familias de moléculas: las tiahuramidas y las laxaficinas. Las tiahuramidas A, B y C se obtuvieron de un espécimen de *Lyngbya majuscula* recolectado en la Polinesia Francesa. Las tiahuramidas B y C resultaron ser tóxicas en la línea celular de neuroblastoma humano SH-SY5Y, siendo la última la más dañina. Por ello se analizó el tipo de muerte que estaba produciendo este compuesto mediante citometría de flujo, confirmándose que su toxicidad se debía a un proceso de necrosis. El otro grupo de compuestos derivados de cianobacterias, las laxaficinas, se divide en dos subfamilias en función de su estructura química: laxaficinas tipo A y laxaficinas tipo B. En concreto, se analizaron las actividades de cinco laxaficinas A y seis laxaficinas B, que fueron aisladas de la cianobacteria *Anabaena torulosa*, también procedente de la Polinesia Francesa, con la excepción de dos de los compuestos pertenecientes al tipo B. Dichas moléculas se obtuvieron de un gasterópodo herbívoro, por lo que presentan modificaciones producidas por la biotransformación, pero se consideran también compuestos derivados de la cianobacteria. Con respecto a los resultados de las laxaficinas tipo A, tres de ellas mostraron toxicidad en las células de neuroblastoma, pero en este caso el análisis del tipo de muerte celular por citometría no fue concluyente. Por tanto, se determinó si la disminución observada en la viabilidad celular estaba causada por otro tipo de proceso, la autofagia. Aunque la activación del flujo autofágico tiene como objetivo la supervivencia de la célula en condiciones de déficit de nutrientes y energía, una excesiva autofagia puede producir la muerte celular. El análisis de la expresión de proteínas implicadas en este proceso, como la cadena ligera 3 asociada a microtúbulos (LC3) y diana de rapamicina en mamíferos (mTOR), confirmó que estas laxaficinas estaban desencadenando autofagia. Por otro lado, las laxaficinas tipo B mostraron diferentes actividades biológicas en función de su estructura química. Las laxaficinas B y B3, con estructura cíclica, resultaron ser tóxicas a través de la activación de la apoptosis, mientras que las demás laxaficinas, de estructura acíclica, produjeron autofagia. En este caso los compuestos aumentan la expresión de la proteína quinasa activada por adenosín monofosfato (AMPK), un importante sensor energético celular se activa con niveles bajos de adenosín trifosfato (ATP), provocando la inhibición de procesos energéticamente costosos y la estimulación de rutas celulares que proporcionan intermediarios para mantener los niveles de ATP, como la autofagia. Es destacable que la biotransformación no parece afectar a la actividad de los compuestos, ya que las laxaficinas aisladas del gasterópodo también producen autofagia, lo que sugiere que los metabolitos pueden estar confiriendo alguna ventaja ecológica al animal. Debido a la toxicidad mostrada por las tiahuramidas, las laxaficinas tipo A y las laxaficinas B cíclicas, se descartó su uso como compuestos neuroprotectores. Sin embargo, la activación de la autofagia está relacionada con la neuroprotección cuando no lleva a la muerte celular, ya que representa una vía de eliminación de agregados de proteína, presentes en las enfermedades neurodegenerativas. Por tanto, las laxaficinas acíclicas tipo B presentan potencial terapéutico para estas dolencias, pero debe clarificarse su mecanismo de acción, ya que parecen afectar también al funcionamiento mitocondrial.

Continuando con los compuestos obtenidos a partir de microorganismos, el filo Actinobacteria representa la mayor fuente de compuestos naturales dentro del dominio Bacteria. En particular, el género *Streptomyces* es el más prolífico, con metabolitos con propiedades anti-inflamatorias, antibióticas o antitumorales. Durante esta tesis se determinó el potencial neuroprotector de tres compuestos derivados de estos organismos: las estreptociclinonas A y B, y el caniferolide A. En primer lugar, se evaluó su capacidad antioxidante en un modelo *in vitro* de estrés oxidativo con la línea de neuroblastoma humano SH-SY5Y. Para generar un desequilibrio entre las especies reactivas de oxígeno (ROS) y las defensas antioxidantes, se añadió H_2O_2 a 150 μM durante seis horas a las células. Tras el tratamiento, se monitorizaron diversos parámetros como el funcionamiento de las mitocondrias, el estado de las defensas antioxidantes o los niveles de ROS, con el objetivo de determinar el efecto protector de los compuestos. Los metabolitos secundarios de *Streptomyces* redujeron el estrés oxidativo, aumentando los niveles del antioxidante glutatión (GSH) y neutralizando las moléculas tóxicas derivadas del oxígeno. Estos efectos están mediados por su capacidad para translocar al núcleo el factor de transcripción nuclear 2 relacionado con el factor eritroide-2 (Nrf2), responsable de la expresión de genes de enzimas antioxidantes como las superóxido dismutasas (SODs) o la catalasa (CAT). En vista de estos resultados, se testaron los compuestos en un modelo celular de neuroinflamación con la línea de microglía de ratón BV2. Para estos ensayos, las células se trataron durante una hora con los metabolitos, y después se añadió lipopolisacárido (LPS) a 500 ng/mL durante 24 horas. El LPS es un componente de la membrana de las bacterias Gram-negativas que provoca la activación de la respuesta inflamatoria en la microglía, causando la liberación de moléculas tóxicas como el óxido nítrico o citoquinas, y la estimulación de rutas celulares como el factor nuclear potenciador de la cadena ligera kappa de células B activadas (NFkB) o las proteínas quinasas activadas por mitógenos (MAPKs). La adición de las estreptociclinonas y el caniferolide A produjo una reducción en la liberación de mediadores pro-inflamatorios y en la activación de enzimas implicadas en la cascada neuroinflamatoria. Además, estos compuestos aumentaron los niveles de la citoquina anti-inflamatoria interleuquina-10 (IL-10) y la activación del factor Nrf2, como ocurría en el neuroblastoma, lo que sugiere un efecto inmunomodulador de estas moléculas. Finalmente, se realizó un co-cultivo con ambas líneas celulares, sembrando la microglía en insertos colocados sobre los pocillos con neuroblastoma, que confirmó las propiedades neuroprotectoras de los compuestos de *Streptomyces*, ya que se observó un aumento en la supervivencia de las células neuronales cuando la microglía activada era tratada con estos metabolitos.

Debido a los prometedores efectos observados tanto en el modelo de estrés oxidativo como en el de neuroinflamación, se decidió analizar dos marcadores específicos de la enfermedad de Alzheimer: tau y Aβ. Para determinar el efecto sobre la hiperfosforilación de tau se utilizó una línea de neuroblastoma transfectada con dos mutaciones específicas de las tauopatías, que resultan en un patrón de fosforilación de la proteína en residuos relacionados con la enfermedad de Alzheimer, las células SH-SY5Y-TMHT441. Se analizó la expresión de tau con anticuerpos específicos, obteniéndose un resultado positivo con todos los compuestos, que fueron capaces de reducir la fosforilación de la proteína. Dicha disminución resultó estar producida por su capacidad para inhibir la actividad de las MAPKs. Además, se determinó el efecto de los compuestos sobre la actividad de la enzima β-secretasa 1 (BACE1), que cataliza el paso irreversible en la liberación de la proteína Aβ. En este caso se trata de un ensayo sin células, en el que la estreptociclinona B y el caniferolide A redujeron la actividad de la enzima de forma dosis-dependiente. Por tanto, se seleccionaron estos compuestos para evaluar su efectividad para regular la activación de la microglía con fibras de Aβ. En ambos casos se vio que los niveles de ROS se reducían considerablemente al añadir los metabolitos a las células. Por tanto, se estableció un nuevo co-cultivo con las células transfectadas y la microglía activada con Aβ, y de nuevo ambos compuestos protegieron a las células neuronales. En vista de los resultados obtenidos, se puede concluir que los compuestos de *Streptomyces* tienen un efecto multifactorial, modulan el estrés oxidativo, la neuroinflamación y reducen la fosforilación de tau y la actividad de BACE1, lo que los convierte en moléculas con un gran potencial para el tratamiento de la enfermedad de Alzheimer.

Con respecto a los compuestos naturales aislados de esponjas, se ha analizado la actividad antioxidante de cuatro metabolitos pertenecientes a dos familias en el modelo de estrés oxidativo descrito anteriormente. La makaluvamina J se obtuvo de un ejemplar de *Zyzzya* sp. recogido en las Islas Fiji, mientras que las sarainas 1, 2 y A se aislaron de la esponja mediterránea *Haliclona (Rizhoniera) sarai*. Los animales del filo Porifera han sido tradicionalmente la mayor fuente de metabolitos de origen marino, representando alrededor de un 50% del total de los compuestos aislados a partir de animales. Dado que se trata de organismos sésiles, dependen en gran medida de la liberación de señales químicas, por lo que han desarrollado un gran metabolismo secundario para protegerse de patógenos y depredadores.

La makaluvamina J fue seleccionada para estos ensayos tras determinar el potencial neuroprotector de siete compuestos de su familia en un modelo de estrés oxidativo con neuronas corticales de ratón, en el que dicho metabolito fue el más activo. En la línea neuronal humana, la makaluvamina J también protege a las células frente al daño producido por el H_2O_2, observándose una recuperación del potencial de membrana mitocondrial y de las defensas antioxidantes. El análisis de la expresión del factor Nrf2 confirmó que este efecto se debía a la activación de su translocación al núcleo. Las sarainas también se testaron en este modelo celular. En este caso sólo la saraina A resultó ser neuroprotectora, reduciendo los niveles de ROS, mejorando la función mitocondrial y aumentando la actividad de las SODs. Los efectos positivos de este compuesto se deben también a la activación de la ruta Nrf2-ARE (elemento de la respuesta oxidante), así como a su capacidad para bloquear la apertura del poro de transición mitocondrial (mPTP). El mPTP es un canal no selectivo que se abre en las mitocondrias, conectando la matriz y el citosol, y permitiendo la salida de solutos. El poro se ha relacionado con las enfermedades neurodegenerativas, ya que contribuye a la disfunción mitocondrial y puede llevar a la muerte neuronal.

A continuación, se evaluaron 24 análogos sintéticos de un compuesto natural de esponjas, la gracilina A. Este compuesto pertenece a una familia de moléculas cuyas propiedades neuroprotectoras e inmunosupresoras se habían descrito anteriormente, por lo que se escogió para llevar a cabo una retrosíntesis con el objetivo de identificar los análogos más simples que conservasen la bioactividad de la molécula natural. Concretamente, durante esta tesis doctoral se ha determinado su actividad neuroprotectora. De todos los análogos analizados, los mejores

resultados se obtuvieron con siete de ellos (21a, 27a, 27b, 29a, 21b, 22 y 23c), que fueron capaces de proteger la función mitocondrial y reducir los niveles de estrés oxidativo en las células de neuroblastoma. Es destacable que algunos de los análogos más eficaces, como el 27a y el 27b, tienen estructuras químicas más simples que el compuesto natural y conservan su actividad. Estos siete compuestos se seleccionaron para evaluar si el efecto sobre la mitocondria estaba mediado por la inhibición del mPTP, como ocurre con las gracilinas naturales. Los análogos 27a, 27b, 29a, 21b y 22 bloquearon la apertura del poro, por lo que se decidió analizar su capacidad para alterar la actividad de la ciclofilina D (CypD), la molécula responsable de la iniciación de la apertura del canal. Los cinco compuestos inhibieron a la enzima a concentraciones bajas, destacando el 27a, el 27b y el 29a, que mostraron selectividad por esa enzima frente a la ciclofilina A (CypA). Esta isoforma está presente en el citosol e interviene en la inmunosupresión. La gracilina A inhibe ambas enzimas a concentraciones similares, de ahí sus propiedades neuroprotectoras e inmunosupresoras, por lo que los compuestos sintéticos presentan esta característica diferenciadora frente al metabolito natural. En concreto, el compuesto 27b mostró la mayor selectividad, lo que se comprobó testándolo en ensayos preliminares de inmunosupresión, en los cuales no presentó ninguna actividad. Esta característica es muy destacable, puesto que apenas se han descrito compuestos con este tipo de selectividad, que les confiere un gran potencial como posibles fármacos para el tratamiento de la enfermedad de Alzheimer. Tras estos ensayos, se determinó la capacidad de los siete análogos para modular la expresión de genes antioxidantes. Todos los compuestos aumentaron la expresión de alguna de las enzimas analizadas (SODs, CAT, Nrf2, glutatión peroxidasa), destacando de nuevo el 27b, que actúa sobre todos los genes. En vista de estos prometedores resultados, se llevaron a cabo ensayos en el modelo de neuroinflamación con la línea de microglía. Los compuestos sintéticos presentaron propiedades inmunomoduladoras a través de la inhibición de NFkB y la activación de Nrf2, con la consecuente reducción en la liberación de moléculas pro-inflamatorias (NO, interleuquina-1β,…). Además, el tratamiento de la microglía con los análogos redujo la muerte de células neuronales en el sistema de co-cultivo descrito anteriormente. De entre los siete compuestos, el 27a y el 27b fueron los más eficaces en el modelo de neuroinflamación. Por tanto, teniendo en cuenta los resultados anteriores, estos análogos, con estructuras más simples que la gracilina A, son los compuestos sintéticos con mayor potencial neuroprotector.

Por último, se han realizado ensayos con 15 bebidas de frutas con el objetivo de determinar su potencial antioxidante y anti-inflamatorio. Se trata de cuatro zumos carbonatados (coco, aloe, açai y manzana), tres mezclas de vino (sangría, tinto de verano y tinto de verano con limón), dos mostos (blanco y tinto) y seis sidras (pera, dulce, seca, fresa, mora y natural) obtenidas de una empresa local. En primer lugar, se analizó su actividad antioxidante en las células de neuroblastoma humano, para lo que se extrajo el contenido polifenólico de las bebidas. Los resultados fueron muy positivos, ya que todos los extractos redujeron el estrés oxidativo, aumentando las defensas antioxidantes celulares. A continuación, se trataron las células de microglía con los extractos obtenidos de las bebidas. En este caso el efecto fue menor, pero el tratamiento con los zumos, las mezclas de vino, y las sidras dulce y de pera redujo la activación de las células inflamatorias, disminuyendo la citoquina pro-inflamatoria factor de necrosis tumoral-α (TNF-α) y aumentando la molécula anti-inflamatoria IL-10. En base a estos datos se escogieron cuatro bebidas para usarlas en un modelo murino de inflamación: el zumo

carbonatado de manzana y las sidras dulce, seca y de pera. Éstas se concentraron, evaporándose el alcohol en el caso de las sidras, y se proporcionaron a los ratones durante una semana en sustitución del agua de bebida. El último día de tratamiento, se provocó una inflamación sistémica a los ratones inyectándoles LPS intraperitonealmente. Tras esto, se determinó el estado de las defensas antioxidantes en el cerebro de los ratones. Todas las bebidas aumentaron considerablemente la expresión de genes antioxidantes, pero curiosamente, al analizar la actividad de las enzimas correspondientes, ésta disminuía con el tratamiento. Esto podría deberse a que los polifenoles que componen las bebidas neutralizan las especies reactivas de oxígeno directamente, por lo que la activación de los sistemas antioxidantes no sería necesaria. También se observó una disminución en los niveles de peroxidación lipídica en el cerebro, con excepción del tratamiento con sidra seca. En cuanto a los marcadores de inflamación, las bebidas de manzana redujeron la liberación de NO en el cerebro, así como los niveles de TNF-α tanto en este órgano como en la sangre. Se observa una diferencia en cuando a la efectividad de los tratamientos, siendo el zumo de manzana y la sidra de pera los más beneficiosos, seguidos por la sidra dulce. En cambio, con el consumo de sidra seca no se obtuvieron efectos positivos.

Los compuestos analizados durante esta tesis doctoral han mostrado efectos neuroprotectores, con la excepción de los derivados de cianobacterias, lo que los convierte en candidatos para una investigación más extensa en el campo de las enfermedades neurodegenerativas, de las cuales el Alzheimer es la patología más común. En concreto, los compuestos aislados de *Streptomyces*, con sus propiedades multidiana, y los análogos sintéticos de la gracilina A, con su selectividad hacia la CypD y su capacidad de modular la inflamación y los genes antioxidantes, son las moléculas más prometedoras para un uso terapéutico. Por otro lado, el consumo de bebidas funcionales de frutas, y más particularmente, de aquéllas derivadas de la manzana, podría ayudar a prevenir la enfermedad de Alzheimer, ya que se han observado efectos neuroprotectores y anti-inflamatorios. La combinación de ambas estrategias, tanto la preventiva como la farmacológica dirigida a la mitocondria y la inflamación, podría resultar beneficiosa para el tratamiento de la enfermedad de Alzheimer, cuyo freno supone un gran reto para nuestra sociedad.

1. INTRODUCTION

Nature has been a source of medicinal compounds for millennia. Natural products are structurally complex molecules that present a great specificity to their biological targets. Plants have historically been the major source of bioactive compounds, until the early 1900s, 80% of all medicines were obtained from those organisms. In particular, dietary products such as fruits, vegetables, beverages and spices are a rich source of active compounds with an interesting potential for prevention and treatment of diseases. Over the last 25 years, two-thirds of developed drugs have derived from natural products, with almost all of these drugs having terrestrial origin. However, the marine environment has emerged as a great source of new bioactive compounds. Advances in ocean exploration, such as scuba diving techniques and remotely operated vehicles have opened the sea to scientific research (Alves et al., 2018; Berkov et al., 2014).

1.1. MARINE COMPOUNDS

Oceans cover the 70% of Earth's surface, harbouring a great biological diversity and a significant potential for the discovery of new bioactive compounds. Compared to terrestrial habitats, marine environment supports a higher diversity at taxonomic level. The oceans house 34 of 36 known animal phyla, with 15 phyla being exclusively marine (Snelgrove, 2016). It has been recently estimated the existence of approximately 2.2 million of eukaryotic marine species, suggesting that about 91% of the oceanic species remains undescribed (Mora et al., 2011). This great diversity of the marine environment is reflected into a bigger pool of structurally diverse bioactive molecules.

Marine organisms have stablished complex intra- and inter-specific interactions, which are in many cases mediated by chemical signals. These interplays have a key role in ecological processes such as competition, commensalism, feeding, or escape strategies, influencing the community structure and the ecosystem function (Hay, 2009).

Particularly, many marine sessile invertebrates, without physical defences, depend on the release of chemical cues to stop predators or paralyze prey (Leal et al., 2012). These molecules are called secondary metabolites because they are not synthetized through the main metabolic pathways (Cassier-Chauvat et al., 2017).

Compounds are usually limited to a taxon (family, genus or even species) and exhibit unique chemical structures. The need for new substances for human diseases treatment, along with the knowledge that marine organisms are great sources of novel compounds, has led to an intensive research. Alkaloids, terpenoids, peptides, steroids and many other bioactive molecules have been isolated from marine organisms.

In the last 50 years, over 30000 marine natural products have been reported (Figure 1). In particular, in the last decade the number of new isolated compounds has increased until 1000 per year (Alves et al., 2018; Blunt et al., 2018).

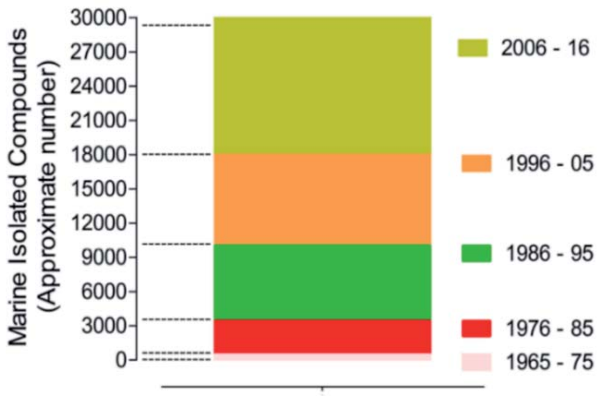

Figure 1. Marine natural products isolated in the last 50 years (approximate number/10 years) (Alves et al., 2018)

From the discovery of a new drug until its approval for medical use it takes about 20 or 30 years. The pharmaceutical pipeline comprises three steps: biodiscovery, development and market phase. The biodiscovery stage includes the isolation of the compound and the identification of the molecular target. Then, the drug goes into the development stage that comprises the clinical trials: in phase I drug's safety is tested in humans, in a small healthy group; in phase II the efficacy and side effects of the compound are determined, and compared to a placebo or a standard approved drug; and finally, the molecule enters phase III, in which the drug is tested in a bigger group of patients for a longer period of time and compared to a control group. Finally, data are compiled and a New Drug Approval dossier is sent to the regulatory agencies (Calado et al., 2018).

To date, eight drugs with marine origin have been approved for clinical use by the Food and Drug Administration (FDA) in the USA or the European Medicine Agency (Figure 2).

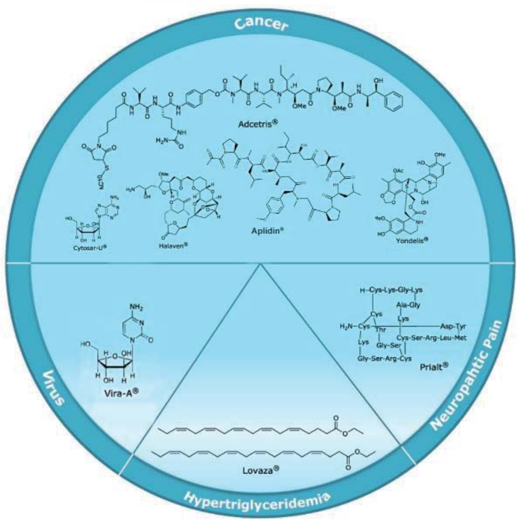

Figure 2. Marine-derived drugs approved for medical use. Modified from (Martins et al., 2014)

The first marine-derived drugs were approved in the 70s: the anti-leukemia cytarabine (Cytosar®) and the antiviral vidarabine (Vira-A®), synthetic derivatives of the nucleosides spongothymidine and spongouridine, isolated from the sponge *Tectitethya crypta*. In 2004, ziconotide (Prialt®) was approved by the FDA for the management of chronic pain. This compound is the synthetic equivalent of the peptide ω-conotoxin MVIIA produced by the gastropod *Conus magus*. In the same year, the FDA also approved a mixture of ω-3-fatty acids ethyl esters derived from fishes (Lovaza®) to reduce triglyceride levels. In the last decade, three new anti-cancer agents with marine origin have been approved: the macrolide eribulin mesylate (Halaven ®), a synthetic analogue of the compound halichondrin B produced by the sponge *Halichondria okadai*; the antibody-drug conjugate brentuximab-vedotin (Adcetris ®), which includes a synthetic derivative of the natural compound dolastatin 10, isolated from the gastropod *Dolabella auricularia*; and the drug trabectedin (Yondelis ®) obtained from the tunicate *Ecteinascidia turbinate* (Altmann, 2017; Calado et al., 2018; Martins et al., 2014). In addition, the cyclic depsipeptide plitidepsin (Aplidin®), isolated from a Mediterranean tunicate, has been recently approved for cancer treatment by the FDA (Alonso-Alvarez et al., 2017).

Currently, six marine-derived compounds are in phase III clinical trials, 14 in phase II and ten in phase I (Mayer, 2019). Therefore, it is expected that more compounds with marine origin reach the market in coming years.

During the present doctoral thesis, the biological activities of 45 marine-derived compounds have been evaluated: 17 were obtained from microorganisms, four from sponges, and 24 compounds are synthetic derivatives of a natural compound isolated from a sponge (Table 1). Thus, the following sections are focused on compounds derived from marine bacteria and sponges.

Table 1. Marine compounds evaluated in this doctoral thesis

Origin	Compounds
Cyanobacteria	Tiahuramides
	Laxaphycins
Actinobacteria	Streptocyclinones
	Caniferolide A
Porifera	Makaluvamine J
	Sarains
	Gracilin A derivatives

1.1.1. Compounds isolated from marine microorganisms

It is estimated that one milliliter of seawater contains 10^2 fungi, 10^3 bacteria and 10^7 viruses (Agrawal et al., 2017), and that one billion species of microorganisms remain undescribed (Snelgrove, 2016). Marine microbes are present in all types of ecosystems from the polar ice to the hydrothermal vents, and from the deep sea to coastal waters. Microbial communities are also associated with the surface of macroorganisms such as algae, sponges and corals (Imhoff et al., 2011). Despite this great abundance and diversity, natural products obtained from microorganisms only represent 19% of the totality of marine compounds (Calado et al., 2018) (Figure 3).

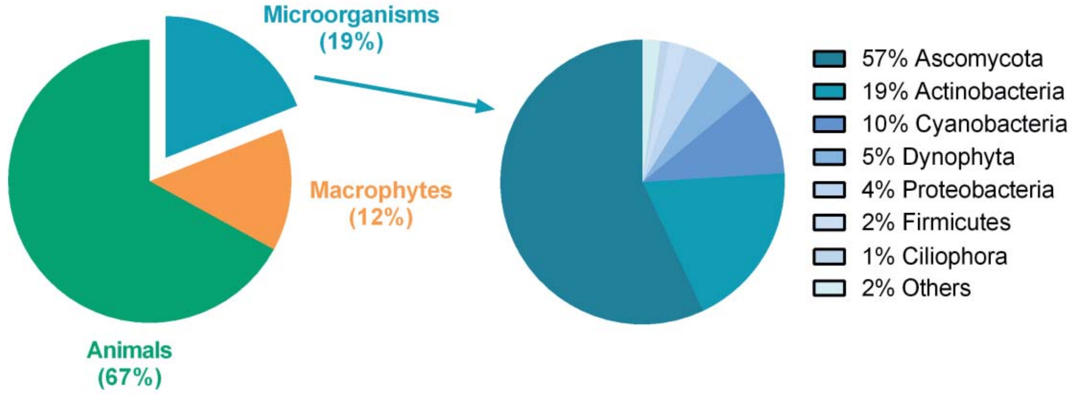

Figure 3. Natural products isolated from marine microorganisms between 1940 and 2015. Modified from (Calado et al., 2018)

Taxonomic limitations and the difficulty for sampling and cultivation are responsible of this low percentage. However, the development of new culturing techniques and genetic tools has increased the interest in compounds derived from these small organisms (Bhatnagar and Kim, 2010; Zhang et al., 2016a). In 2000s, Ascomycota and Bacteria emerged as producers of bioactive compounds and their proportional contribution to marine natural products in the last years has surpassed other phyla as Porifera and Cnidaria, traditionally associated with the production of bioactive compounds (Snelgrove, 2016).

As Figure 3 shows, more than a half of marine microorganisms-derived compounds are produced by fungus (Ascomycota), followed by Actinobacteria (19%) and Cyanobacteria (10%). A brief description of the last two phyla is presented below, as the microbe-derived compounds tested in this thesis were isolated from members of these taxa.

The phylum Cyanobacteria includes photosynthetic prokaryotes from terrestrial, freshwater and marine ecosystems. These Gram-negative organisms, also called blue-green algae, appeared on Earth over 3.5 billion years ago and can live as colonial or unicellular forms in almost every habitat (deserts, ice shelves, as endosymbionts…). The high degree of adaptation of cyanobacteria is related to their ability to produce a wide range of secondary metabolites (Liu and Rein, 2010; Rastogi and Sinha, 2009). The majority of these compounds are complex peptides, depsipeptides or lipopeptides that contain unusual amino acids and multiple N-methylations (Vinothkumar and Parameswaran, 2013), and have displayed antibacterial, antiviral, antifungal and cytotoxic activities (Agrawal et al., 2017; Burja et al., 2001; Tan, 2007). These molecules are produced through non-ribosomal peptide synthase (NRPS) and/or polyketide synthase (PKS) enzymatic systems (Agrawal et al., 2017). These enzymes are organized in modules, each one carrying out the addition of a substrate to the molecule, generating a great diversity of compounds (Condurso and Bruner, 2012; Jones et al., 2010).

The phylum Actinobacteria is one of the largest taxonomic units within the domain Bacteria. Actinobacteria are Gram-positive bacteria distributed in terrestrial and oceanic environments. The majority are free-living organisms with an extensive secondary metabolism. In fact, two-thirds of the natural-derived antibiotics in clinical use are produced by actinomycetes (Barka et al., 2016). In the ocean, these microbes are present in the sediments, intertidal zones, in the water column and associated with macroorganims. They contribute to the recycling cycle of organic matter, fixation of nitrogen and immobilization of nutrients (Subramani and Aalbersberg, 2012).

Next, the microbe-derived compounds that have been evaluated in the present doctoral thesis are described, ordered by their origin. Firstly, cyanobacteria-derived compounds are presented, followed by molecules isolated from Actinobacteria.

- Tiahuramides

Tiahuramides are a family of three cyclohexadepsipeptides isolated from the marine filamentous cyanobacteria *Lyngbya majuscula*. The genus *Lyngbya* includes species distributed worldwide, formed of cylindrical filaments that usually produce large mats and blooms in fresh and sea water. In particular, *Lyngbya. majuscula* is the most important species in terms of secondary metabolites discovery (Liu and Rein, 2010). The cyanobacteria produces cyclic peptides such as lyngbyastatins (Williams et al., 2003), apratoxin A (Luesch et al., 2001) or lyngbyabellins (Luesch et al., 2000a; Luesch et al., 2000b). Within these lipopeptides, there are cyclic penta, hexa, and heptadepsipeptides such as yanucamides (Sitachitta et al., 2000) or hantupeptins (Tripathi et al., 2010).

Tiahuramides A, B and C (Figure 4a) were isolated from a collection of *Lyngbya majuscula* at Tiahura Atoll, Moorea Island (French Polynesia) (Figure 4b). The compounds are cyclohexadepsipeptides, new members of the NRPS-PKS peptide family. Specifically, tiahuramides belong to a sub-family that contains a fatty acid moiety within an overall cyclic depsipeptide framework (Boudreau et al., 2012).

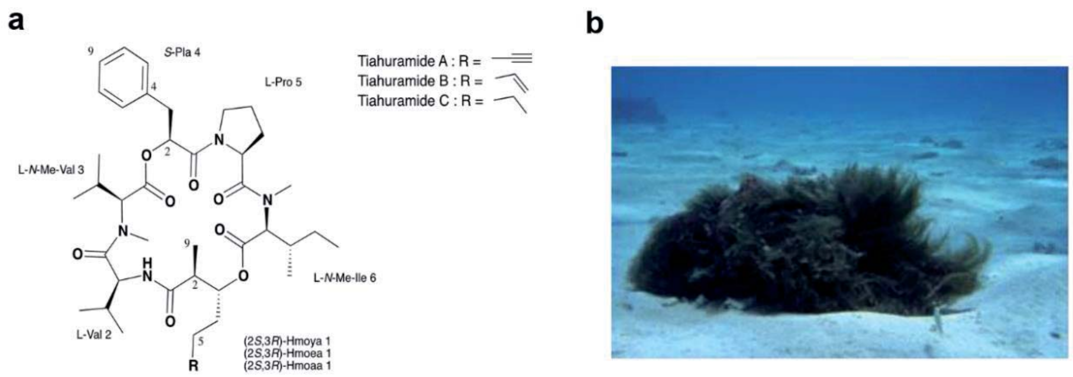

Figure 4. (a) Chemical structures of tiahuramides A, B and C (b) Specimen of the cyanobacteria *Lyngbya majuscula*. Photograph courtesy of Prof. Bernard Banaigs

- Laxaphycins

Laxaphycins are a family of lipopeptides isolated from several cyanobacteria species found worldwide. These compounds are characterized by the presence of non-proteinogenic amino acids and are divided in two sub-families: laxaphycin A-type compounds, undecapeptides with a segregation of hydrophobic and hydrophilic residues; and laxaphycin B-type peptides, dodecapeptides in which hydrophobic and hydrophilic residues are alternated (Banaigs et al., 2014).

Laxaphycins were isolated for first time from the freshwater species *Anabaena laxa* (Frankmolle et al., 1992a). Since then, several congeners have been described: hormothamnin A (Gerwick et al., 1989), laxaphycins B2 and B3 (Bornancin et al., 2015), lobocyclamides (MacMillan et al., 2002), lyngbyacyclamides (Maru et al., 2010), trichormamides (Luo et al., 2015; Luo et al., 2014) and laxaphycins A2 and B4 (Cai et al., 2018). These compounds have been obtained from *Hormothamnion enteromorphoides*, *Anabaena torulosa*, *Lyngbya confervoides, Lyngbya* sp., *Trichormus* sp. and *Oscillatoria* sp., respectively. The fact that different species could produce similar metabolites suggests that there is horizontal gene transfer or an ancient common parent between them. Therefore, these peptides have been selected by evolution in both freshwater and oceanic cyanobacteria and may confer them an ecological advantage (Banaigs et al., 2014).

The biological activities of laxaphycins have been assayed in some *in vitro* models. Regarding laxaphycin A-type subfamily, the compounds did not show antimicrobial or antifungal activities (Frankmolle et al., 1992b), and only hormothamnin A presented half maximal inhibitory concentration (IC_{50}) values in the micromolar range against cancer cell lines, whereas other compounds from this sub-family displayed a weaker activity or were inactive in these assays (Banaigs et al., 2014; Luo et al., 2014). On the other hand, laxaphycin B-type members such as laxaphycins B, B2, B3, lyngbyacyclamides and lobocyclamides presented higher cytotoxic, antifungal and antimicrobial activities (Bonnard et al., 2007; Cai et al., 2018; Frankmolle et al., 1992b; MacMillan et al., 2002). Interestingly, the combination of laxaphycins A and B produced a synergistic effect, enhancing the toxicity of laxaphycin B (Bonnard et al., 2007; Frankmolle et al., 1992a). This synergism has been recently confirmed with the mixture of laxaphycin A and laxaphycin B4 (Cai et al., 2018).

A collection of *Anabaena torulosa* (Figure 5a) during a bloom in the lagoon of Moorea (French Polynesia) resulted in the isolation of four new A-type laxaphycins: three acyclic compounds and one cyclic.

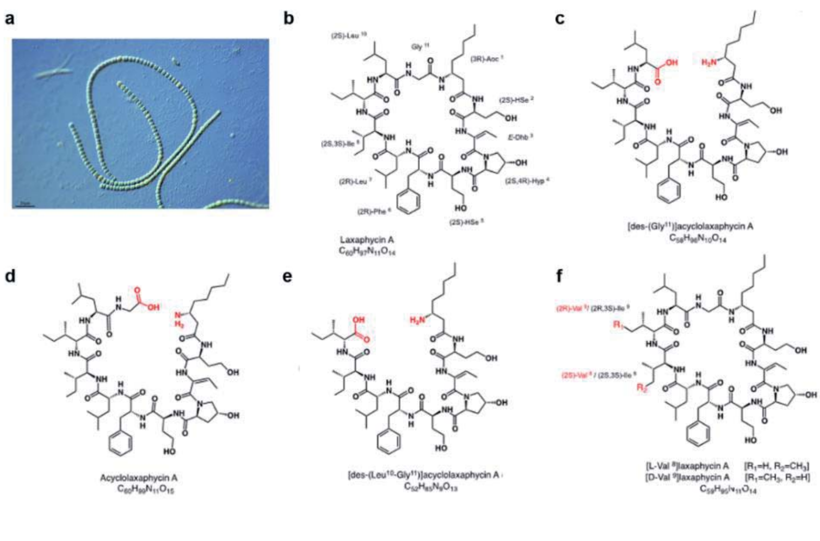

Figure 5. (a) Strain of *Anabaena torulosa* (b-f) Structures of laxaphycins A-type peptides. Modifications with respect to laxaphycin A are highlighted in red

The new compounds were named [des-Gly[11]] acyclolaxaphycin A, acyclolaxaphycin A, [des-(Leu[10]-Gly[11])] acyclolaxaphycin A, and [D-Val[9]] laxaphycin A. The recently described compound laxaphycin A2 ([L-Val[8]] laxaphycin A) (Cai et al., 2018) was also obtained from this collection (Figure 5c-f). This is the first description of acyclic analogues in this family of peptides.

Moreover, four laxaphycin B-type peptides were obtained from the same specimen: the cyclic laxaphycins B and B3, and the acyclic compounds acyclolaxaphycins B and B3 (Bornancin et al., 2015) (Figure 6a-d).

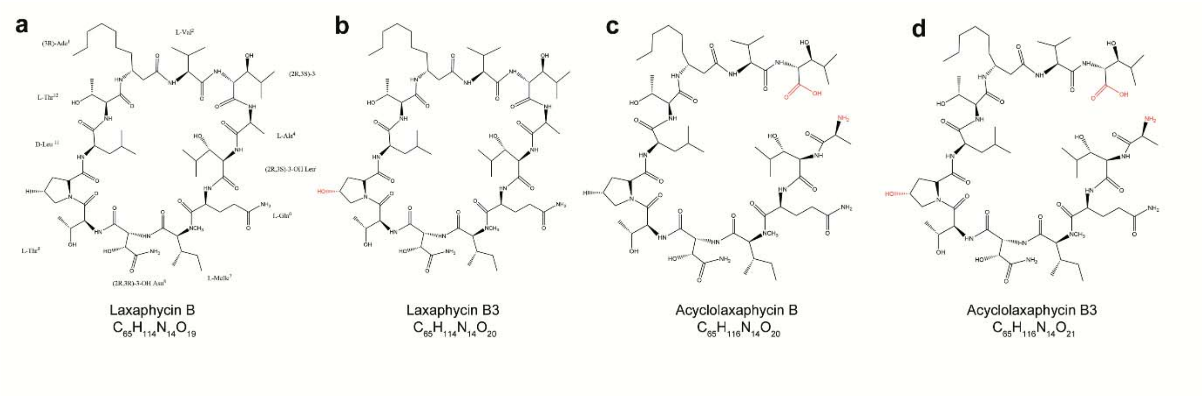

Figure 6. Chemical structures of laxaphycin B-type peptides obtained from *Anabaena torulosa*. Modifications with respect to laxaphycin B (a) are highlighted in red

Interestingly, two new acyclic B-type laxaphycins were obtained from the herbivorous gastropod *Stylocheilus striatus* (Figure 7). This is the first description of diet-derived laxaphycins, which present structural modifications due to biotransformation.

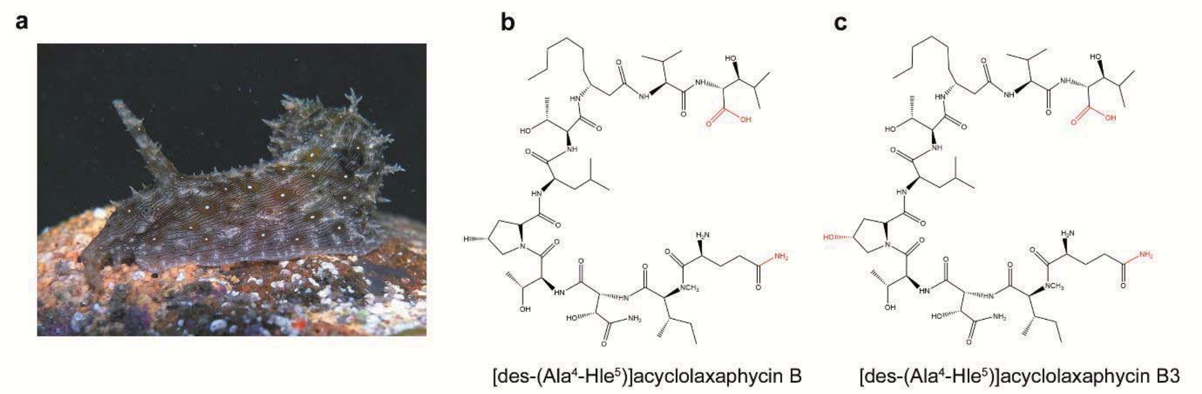

Figure 7. (a) Specimen of *Stylocheilus striatus*. Photograph by Sylke Rohrlach (https://www.flickr.com/photos/87895263@N06/17108069750) licensed by CC BY-SA 2.0. (b-c) Structures of new acyclic B-type laxaphycins

- Streptocyclinones and caniferolide A

The genus *Streptomyces* represents about an 80% of compounds isolated from marine actinomycetes. Streptomycetes are a big source of bioactive metabolites such as peptides, angucyclinones, macrolides or lactones (Subramani and Aalbersberg, 2012) with antibiotic, anti-inflammatory, antifungal or antioxidant properties (Hassan et al., 2017; Hassan and Shaikh, 2017; Subramani and Aalbersberg, 2012).

The cultivation of *Streptomyces* sp CA-237351 led to the isolation and characterization of two new glycosylated angucyclinones, streptocyclinones A and B (Figure 8).

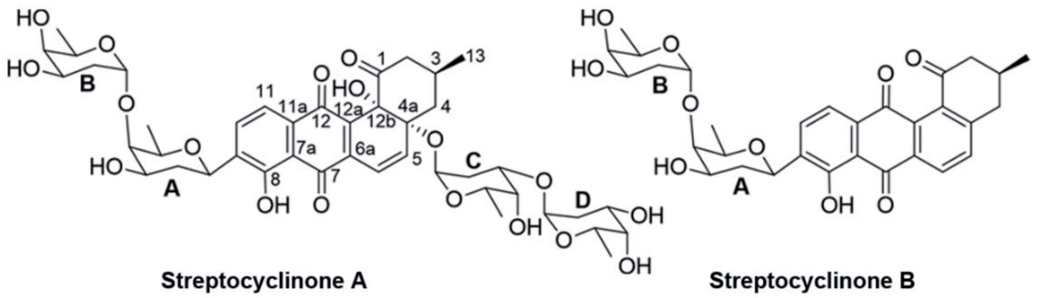

Figure 8. Chemical structures of streptocyclinones A and B

These molecules share structural characteristics with urdamycins, a family of compounds obtained from different *Streptomyces* spp. with cytotoxic and antibiotic activities (Drautz et al., 1986; Ueda et al., 2011).

Otherwise, the macrolide caniferolide A (Figure 9a) was obtained from the marine-derived actinomycete *Streptomyces caniferus* (CA-271066) (Figure 9b). The bacteria was isolated from an ascidian collected in Bahía Ana (São Tomé and Principe) and cultured following a scaled-up fermentation.

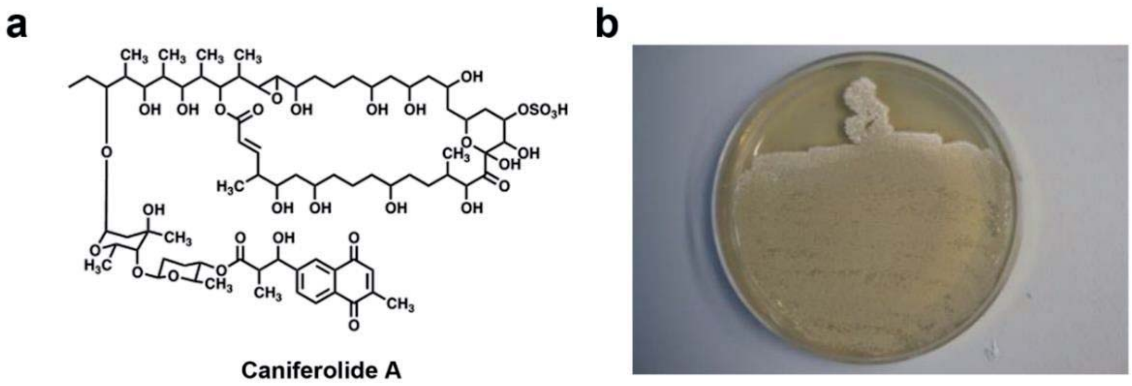

Figure 9. (a) Chemical structure of the macrolide. (b) Culture of *Streptomyces caniferus* (CA-271066). Photograph courtesy of Fernando Reyes

A bioassay-guided isolation led to the purification of three new 36-membered macrolides and an already known one, GT-35, an antifungal macrolide reported in a Japanese patent (Takahashi et al., 1997). The structural elucidation revealed that caniferolide A was the 19-*O*-sulphate derivative of GT-35 (Perez-Victoria et al., 2019). The *Streptomyces*-derived metabolite is structurally related to PM100117 and PM100118 (Perez et al., 2016), axenomycins (Arcamone et al., 1973a; Arcamone et al., 1973b; Sora et al., 1980) and deplelides (Takeuchi et al., 2017), which have shown antitumor, antibiotic and antifungal activities. The compound has displayed antifungal activities against *Candida albicans* and *Aspergillus fumigatus* with minimal inhibitory concentrations of 1 and 8 mg/mL, respectively (Perez-Victoria et al., 2019).

1.1.2. Compounds isolated from marine sponges

The majority of marine compounds isolated until now have been obtained from animals (Figure 10). The main reason is that bioprospecting efforts have been focused on macroorganisms (animals and macrophytes) because of their easier accessibility and taxonomical identification (Calado et al., 2018).

As can be seen in Figure 10, secondary metabolites produced by invertebrates represent the majority of marine natural products isolated until now. Specifically, sponges (phylum Porifera) alone account for almost a half of new compounds since 1940. Organisms from the phylum Cnidaria, particularly corals and jellies, contribute to a 30% of total bioactive compounds. Secondary metabolites have also been obtained from other invertebrates such as echinoderms, molluscs, polychaetes and crustaceans, but represent a smaller proportion (Calado et al., 2018; Snelgrove, 2016).

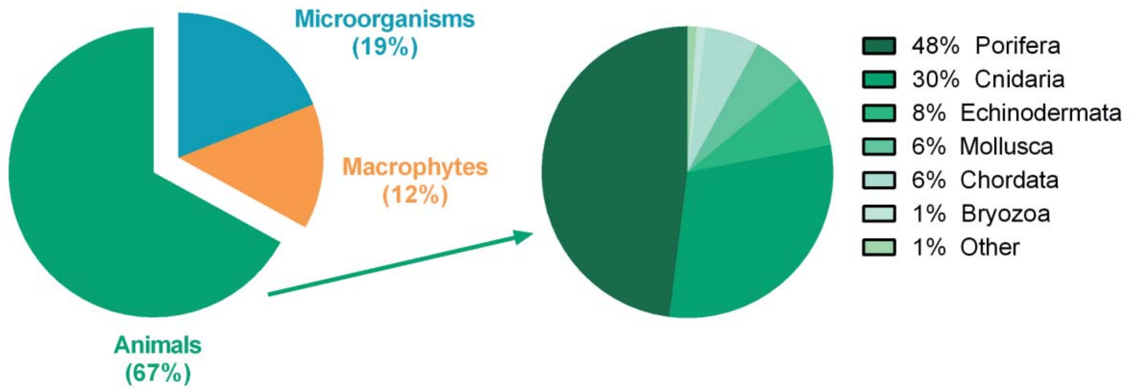

Figure 10. Compounds isolated from marine animals between 1940 and 2015. Modified from (Calado et al., 2018)

The phylum Porifera comprises about 8500 species of filter feeders with small pores in their outer walls connected through aquiferous canals. Sponges have flagellated cells (choanocytes) that move seawater and retain particulate matter and microorganisms (Steiner et al., 2018). Diverse microbial communities are present in sponges, which could account for about 38% of total biomass, and are known to produce several bioactive compounds (Bibi et al., 2017). Also, as mentioned before, sponges have a big secondary metabolism due to their sessile nature, which has caused them a great dependence on the release of chemical cues. Secondary metabolites protect them from predators and pathogens, and provide advantages for space competition (Anjum et al., 2016).

Many kinds of compounds have been isolated from sponges: alkaloids, terpenoids, nucleosides, phenols, polyketides, peptides (Mioso et al., 2017). In fact, three synthetic analogues of sponge-derived molecules have been approved for medical use, and one compound obtained from the sponge *Lithoplocamia lithistoides*, plocabulin, is currently in phase II clinical trials for treatment of advanced solid tumors (Gomes et al., 2018; Mayer, 2019).

In this doctoral thesis, the neuroprotective activities of four compounds obtained from these sessile organisms and 24 synthetic derivatives of a sponge compound have been determined. Their chemical structures and main biological activities are described below.

- Makaluvamines

The makaluvamines are a group of alkaloids isolated mainly from marine sponges of the genus *Zyzzya* (Barrows et al., 1993; Schmidt et al., 1995). These compounds belong to a family of metabolites characterized by a pyrroloiminoquinone skeleton. About 60 sponge-derived molecules including isobatzellines, discorhabdins, tsitsikammamines, or epinardins form this family (Botic et al., 2017; Guzman et al., 2009; Kita and Fujioka, 2012; Li et al., 2018a). Particularly, makaluvamines present structures based in a 7-amino substituted pyrroloiminoquinone ring with common substitutions at N-1, N-5 and N-9 (Antunes et al., 2005).

The cytotoxic activities of makaluvamines against tumor cell lines have been studied. This family of compounds inhibited the DNA processing enzyme topoisomerase II, showing IC_{50} values in the micromolar range (Barrows et al., 1993; Casapullo et al., 2001; Venables et al., 1997), with makaluvamine H as the most potent molecule (Dijoux et al., 2005). Moreover, makaluvamines J, G and L displayed antimalarial activity (Davis et al., 2012) and makaluvamines C and G inhibited nicotinic receptors (Kudryavtsev et al., 2014; Kudryavtsev et al., 2018). On the other hand, five makaluvamines (C, E, G, H and L) have been tested in a cell-free antioxidant assay, being compounds G and E the most effective ones (Utkina, 2013).

A collection of a *Zyzzya* sp. in Fiji Islands led to the isolation of seven makaluvamines (A, F, G, H, J, K and P) (Figure 11). In the present doctoral thesis, the antioxidant-mediated neuroprotective effects of makaluvamine J have been determined in an *in vitro* model of oxidative stress.

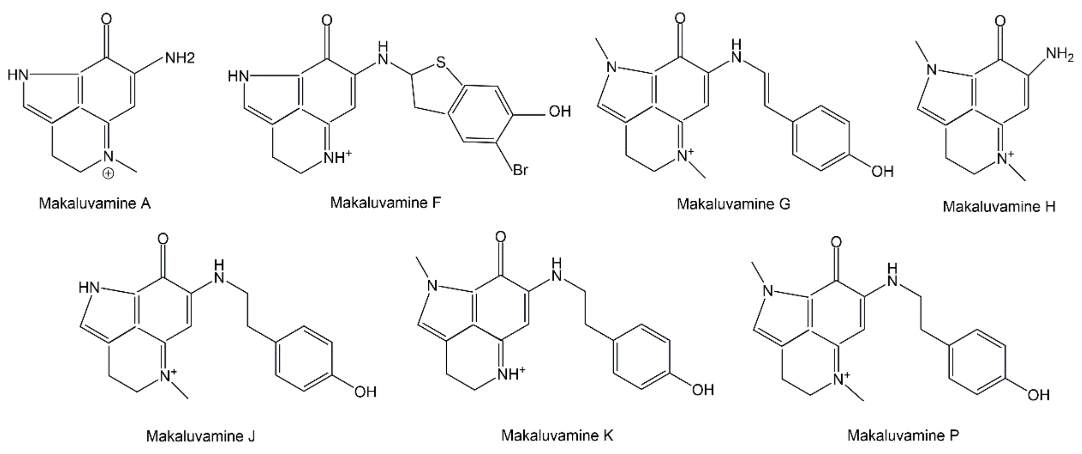

Figure 11. Chemical structures of makaluvamines

- Sarains

The sarains, also called saraines, were obtained for first time in 1986 from the sponge *Reiniera sarai* (re-classified as *Haliclona (Rhizoniera) sarai*), collected in the Bay of Naples (Cimino et al., 1986). In this study, Cimino described the structure of sarains 1, 2 and 3, pentacyclic metabolites that belong to the class of 3-alkylpiperidine alkaloids isolated from sponges. In 1989, three new compounds with a different skeleton were isolated from the same group, sarains A, B and C (Cimino et al., 1989b). The structural elucidation of these compounds took many time due to their complexity (Cimino et al., 1989a; Cimino et al., 1990; Guo et al., 1996b).

Other compounds, such as isosarains 1, 2 and 3, minor co-occurring isomers of sarains 1-3 (Cimino et al., 1991; Cimino et al., 1989c; Guo et al., 1996a), and misenine (Guo et al., 1998) were isolated from the same sponge extract. More recently, all the compounds were obtained from a specimen of *Haliclona sarai* collected in Northern Adriatic Sea (Defant et al., 2011).

Of these ten compounds, sarain A has focused the interest of synthetic chemists because of its unique structure, with an unusual interaction between a tertiary amine and an aldehyde (Delpech, 2014). Several attempts were carried out to achieve the synthesis of the tricyclic core of the compound (Hourcade et al., 2018; Moo Je Sung et al., 1999; Price Mortimer et al., 2008; Sisko and Weinreb, 1991; Yang and Huang, 2010), and finally the total synthesis was reported in 2006 (Becker et al., 2007; Garg et al., 2006).

Despite the great efforts in structure elucidation and synthesis of sarains, the assays to determine the biological properties of this family of metabolites are scarce. It has been proposed that these compounds play a protective anti-fouling role for the sponge since there is an absence of epibionts on its surface (Delpech, 2014). Sarains 1, 2, 3 and A, B, C exhibited toxicity against the shrimp *Artemia salina*, inhibited the growth of sea urchin eggs, and displayed insecticidal/acaricidal, antibacterial and haemolytic effects, as well as inhibition of acetylcholinesterase (AChE) activity (Caprioli et al., 1992; Defant et al., 2011). Sarain 1 also showed anti-fouling properties against larvae of a barnacle by preventing their settlement (Blihoghe et al., 2011).

In the present doctoral thesis, the neuroprotective effects of sarains 1, 2 and A have been evaluated. The metabolites were obtained from a specimen of *Haliclona sarai* collected in Saint-Jean-Cap-Ferrat (France) (Figure 12).

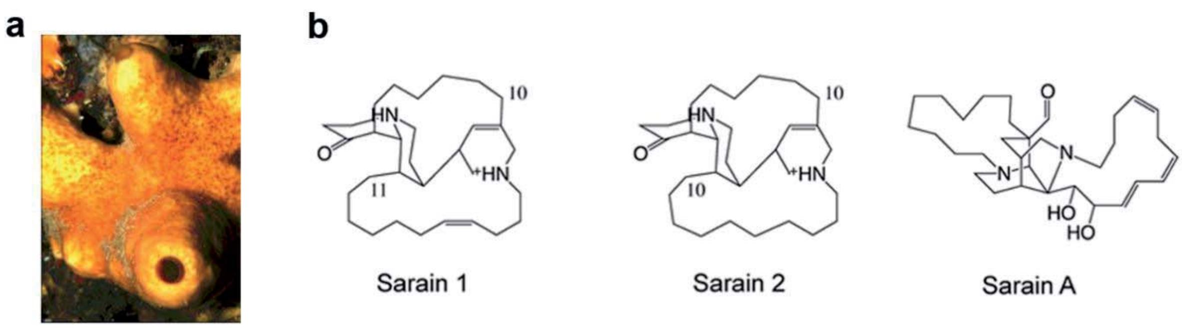

Figure 12. (a) Specimen of *Haliclona (Rhizoniera) sarai* (Garaventa et al., 2010) (b) Chemical structures of sarains 1, 2 and A

- Gracilin A derivatives

The gracilins are a family of diterpenes isolated for first time from the Mediterranean sponge *Spongionella gracilis*. These metabolites are structurally unique due to the diacetoxy furanose found in most members (Figure 13a-f). The first compounds discovered were gracilins A and B (Mayol et al., 1985a, b). One year later, gracilins C-F were described (Mayol et al., 1986a; Mayol et al., 1986b). Then, gracilins G-I were obtained from a specimen of the sponge *Spongionella pulchella* collected in the Canary Islands. Their cytotoxicity, together with gracilins B and C, was tested in a panel of 12 human cancer cell lines. Compounds showed IC_{50} values in the micromolar range. Gracilin B also displayed antiadhesive properties (Rueda et al., 2006).

In 2009, three new gracilins (J-L) and a new diterpenoid (3`-norspongiolactone) were isolated, together with the already known gracilins A, H, I and tetrahydroaplysulphurin-1. These compounds were cytotoxic against leukemia and normal peripheral blood mononuclear human cells, with gracilin A as the most potent molecule (IC_{50}: 0.6 and 0.8 µM, respectively). These gracilins inhibited the epidermal growth factor receptor, a tyrosine kinase associated with different types of human tumors, being gracilin L the most effective compound (Rateb et al., 2009). Moreover, the nor-diterpene gracilin A has been reported as an inhibitor of phospholipase A_2, an enzyme involved in inflammatory diseases (Nirmal et al., 2008).

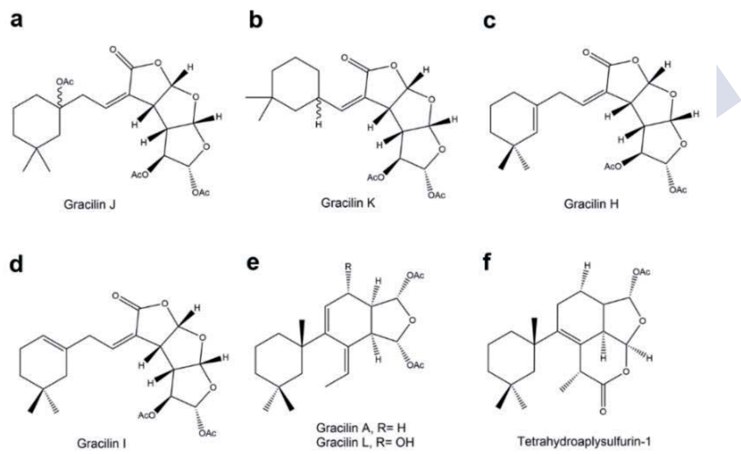

Figure 13. Chemical structures of some compounds of the gracilin family

Interestingly, in 2016 six new gracilin-type metabolites were isolated from the Australian nudibranchs *Goniobranchus splendidus* and *Goniobranchus daphne*. These molluscs eat sponges, and other diterpenes have been reported in sponges of the same region, so the molecules could have a dietary origin. Compounds were screened for cytotoxicity against HeLa S3 cells and gracilin A turned out to be the most active one, with an IC_{50}< 0.3 µg/mL (Hirayama et al., 2016).

Our previous studies revealed that gracilins protect cortical neurons from oxidative stress (Leirós et al., 2014) and block the opening of the mitochondrial permeability transition pore (mPTP) through binding to cyclophilin D (CypD) (Sanchez et al., 2015). Compounds also improved Alzheimer's disease (AD) hallmarks *in vitro* by reducing tau hyperphosphorylation and β-secretase 1 (BACE1) activity. Moreover, gracilins H and L showed promising results in a transgenic mouse model of AD (Leirós et al., 2015). Otherwise, gracilins have also displayed immunosuppressive properties by targeting cyclophilin A (CypA) and reducing interleukin-2 (IL-2) release (Sanchez et al., 2016a). These compounds diminished the expression of the membrane receptor CD147 and presented anti-inflammatory abilities *in vivo* (Sanchez et al., 2016b). Taken together, these results were a good starting point for the use of gracilins as drug leads for a synthetic approach.

Drug development from marine organisms presents a critical bottleneck, the availability of enough quantities of organisms and compounds (Lindequist, 2016). The concentration of pure metabolites in their animal sources is sometimes less than 1 ppm of the wet weight, and the removal of biomass from the ecosystem could have many ecological consequences (Steiner et al., 2018). This supply problem can be solved with aquaculture techniques or with synthetic approaches. In case of sponges, their cultivation has been tried in bioreactors and in-sea aquaculture with disappointing results (Steiner et al., 2018), making the synthetic approach more recommendable.

There are several examples of synthetic compounds based on sponge chemistry (Andersen, 2017), with halichondrin B as the most remarkable. This complex molecule had displayed anticancer properties but its concentration in the sponge was very low. The supply problem was fixed with the discovery that one part of the molecule was responsible of its activity and the following production of the synthetic derivative, which was approved for medical use in 2010 (Newman, 2016).

In view of the promising results (both immunosuppressive and neuroprotective) obtained with gracilin A, this compound was chosen to perform a pharmacophore directed retrosynthesis (PDR). This synthetic strategy could allow to identify simplified versions of the natural product with similar activity potency. Following the PDR strategy, 24 gracilin A derivatives were synthetized (Figure 14). The neuroprotective properties of these synthetic compounds have been objective of this doctoral thesis.

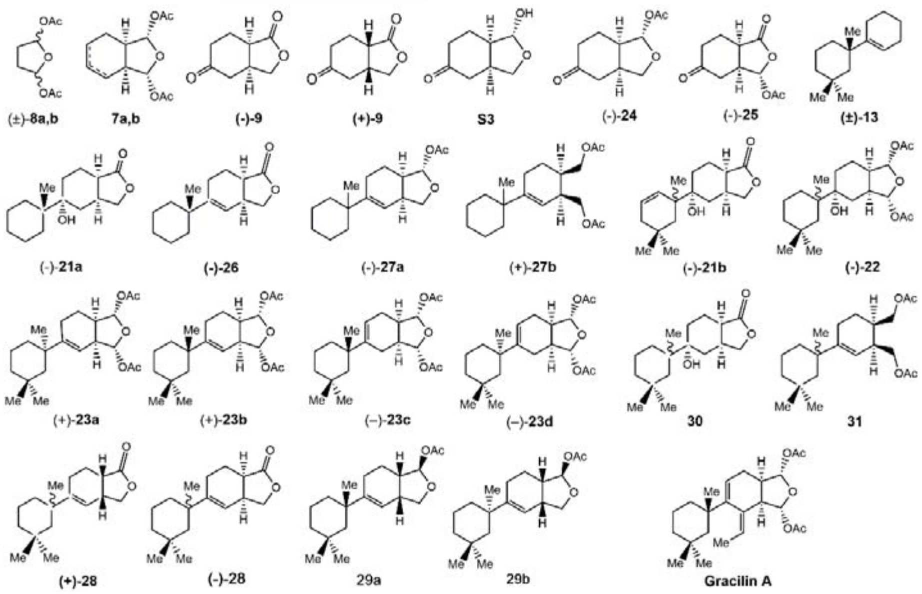

Figure 14. Chemical structures of synthetic derivatives of gracilin A

1.2. BIOACTIVE COMPOUNDS FROM FRUITS AND PLANTS

Plants and fruits are great sources of compounds with biological activities. As mentioned before, organisms from terrestrial habitats have been objective of an intensive research for centuries, being plants the major origin of bioactive compounds until now. In recent years, one class of molecules widely found in plants and fruits has attracted much attention, the polyphenols.

Phenolic compounds share a common structural feature: an aromatic ring bearing at least one hydroxyl substituent. From this simple structure, thousands of compounds have been described. Polyphenols are classified into several subclasses based on their chemical structure: flavonols, chalcones, stilbenes, phenolic acids, flavones, anthocyanins… (Figure 15) (Losada-Barreiro and Bravo-Diaz, 2017). They are involved in defence against ultraviolet radiation, cold temperatures, herbivores or parasites, and contribute to the organoleptic properties of fruits, leaves and berries and their derived products, such as tea, wine, juices, or olive oil (Tresserra-Rimbau et al., 2018).

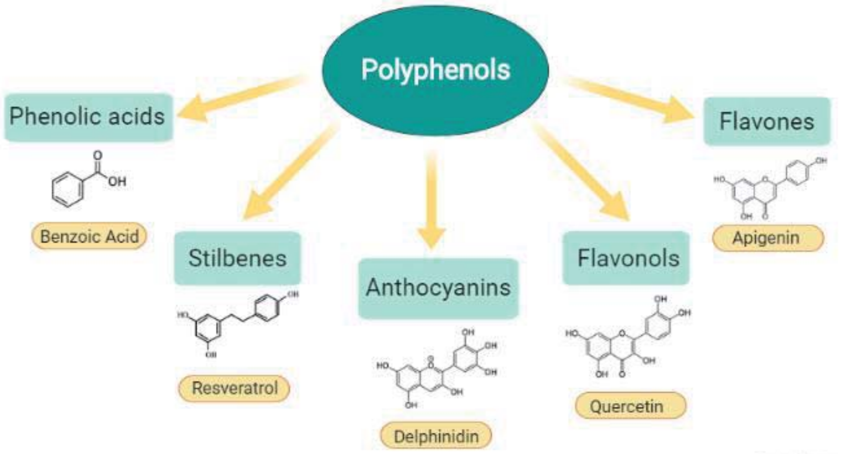

Figure 15. Examples of polyphenolic compounds and their classification

Polyphenolic compounds such as resveratrol, anthocyanins or quercetin have shown beneficial effects against diabetes, cancer or cardiovascular and neurodegenerative diseases, and some of them have entered in clinical trials (Martin et al., 2017; Molino et al., 2016). The main property of polyphenols is their antioxidant capacity. They can directly neutralize damaging molecules by reducing them and have also been described as inducers of cellular antioxidant systems. Moreover, the combined use of two or more polyphenols may produce synergistic effects, so their mixture will generate a higher response than the individual compounds (Renaud and Martinoli, 2019).

Diet is the main source of polyphenols and epidemiological studies have suggested that fruit-rich diets, such as Mediterranean diet are related to a lower risk of suffering cardiovascular or neurodegenerative diseases (Solfrizzi et al., 2011; Zheng et al., 2017). The beneficial effects of this diet include an improvement in redox state and a low grade of inflammation (Mitjavila et al., 2013; Pounis et al., 2016). Particularly, fruit and plant-derived drinks consumption has been related to the prevention of cognitive impairment (Dai et al., 2006), i.e. green tea extract possess anti-inflammatory and antioxidant properties, and blueberry and pomegranate juices improved learning in a mouse model (Islam et al., 2017). Moreover, apple juice has been shown to improve behavioural symptoms in AD patients (Remington et al., 2010) and has presented promising results *in vivo*, preventing cognitive impairment (Chan and Shea, 2006; Ortiz and Shea, 2004; Tchantchou et al., 2005).

In this doctoral thesis, the neuroprotective ability of 15 fruit-derived beverages, produced by a local industry, was determined. Four carbonated juices (cocoa, acai, aloe and apple), three red mixes (sangria, 'tinto de verano', and 'tinto de verano' with lemon), two musts (red and white) and six ciders (pear, sweet, dry, strawberry, blackberry and natural) were used to perform *in vitro* experiments. Then, four apple-derived drinks were supplied to a mouse model of inflammation and their *in vivo* neuroprotective properties were evaluated.

As has been explained in these sections, natural compounds present a wide range of bioactivities. Among them, it is noteworthy their ability to improve mitochondrial function and to regulate oxidative stress and the inflammatory response, two key processes in neurodegeneration. The high brain oxygen consumption and its limited antioxidant defences make it very susceptible to oxidative damage (Nunomura et al., 2006). Moreover, the sustained presence of an oxidative and toxic environment leads to the activation of brain immune cells. Microglial function is dysregulated, and the cells continuously release toxic mediators that contribute to disease progression, since they can produce neuronal death (Maccioni et al., 2018). In this context, natural products can be candidate drugs for neurodegenerative diseases such as AD, the most common dementia. In the following epigraphs, the molecular mechanisms of this illness are described, with a particular focus on oxidative stress and neuroinflammation.

1.3. ALZHEIMER'S DISEASE

AD was described for first time in 1907 (Alzheimer, 1907), but it took around 70 years before it was recognized as the most common cause of dementia (Katzman, 1976). Dementia is a term that refers to a decline in memory, language, attention, reasoning and other cognitive abilities that interfere with daily activities. The loss or damage of neurons in brain regions involved in cognitive function is responsible of this decline (Alzheimer's-Association, 2016). Four stages of AD have been differentiated, each one with characteristic symptoms:

- Preclinical: patients present mild memory loss, not significant, without impairment of their daily activities. Pathological changes begin to appear in the entorhinal cortex, followed by the hippocampus.
- Mild AD: the pathology extends to the cerebral cortex and the cognitive impairment starts. Symptoms include memory loss, inability to remember new information, disorientation, personality changes and disability in reasoning.
- Moderate AD: the illness progresses to areas of the cerebral cortex that control language and sensory processing. Memory and attention loss increases, and behavioural problems begin. Patients have difficulties to recognize their family and present apathy, loss of inhibition and problems in writing and reading.
- Severe AD: the entire cortex is affected by the disease. Patients do not recognize their family and friends. They are completely dependent on others for daily activities (Kumar and Tsao, 2018).

A small number of AD cases are due to inherited gene mutations, only a 1% or less. There are three genes involved in familial AD: those encoding amyloid precursor protein (APP) and presenilin 1 and 2 proteins. People with these mutations usually develop the illness before the age of 65 (Bekris et al., 2010). The remaining 95% of AD patients belong to the so-called sporadic cases. Several risk factors have been associated with the neurodegenerative pathology, such as cardiovascular diseases, traumatic brain injury, smoking, depression, the apoliprotein E ε4 allele, educational level, diabetes… (Kumar and Tsao, 2018). But the greatest risk factor is age, most of AD patients are over 65 years old and as age increases, the probability of developing the pathology augments (the incidence rate doubles every 5 years after the age of 65) (Dubois et al., 2014).

In this context, the increase in life expectancy has augmented the number of AD cases. In US it is estimated that 5.4 million of people suffer this type of dementia, and deaths from AD have augmented 71% in the last 10 years (Alzheimer's-Association, 2016). In Spain, there are around 400 000 cases of AD and it is calculated to reach one million in 2050 (Villarejo Galende et al., 2017). In fact, AD is the second cause of death in our country (Soriano et al., 2018).

Currently, none of the pharmacologic treatments available slows or stops the neuronal damage produced by the illness. These medicaments only provide a temporary improvement of symptoms and their effectiveness varies among patients (Alzheimer's-Association, 2016).

1.3.1. Pathology and molecular mechanism of Alzheimer's disease

AD neuropathology is characterized by the presence of senile plaques and neurofibrillary tangles (NFTs) (Figure 16). The first lesions appear in neurons of the hippocampus and the cortex, areas related to memory and learning. Then the illness progresses to the temporal cortex, the cerebellum and the frontal lobes. These brain regions with plaques and tangles exhibit a reduction in the density of synapses.

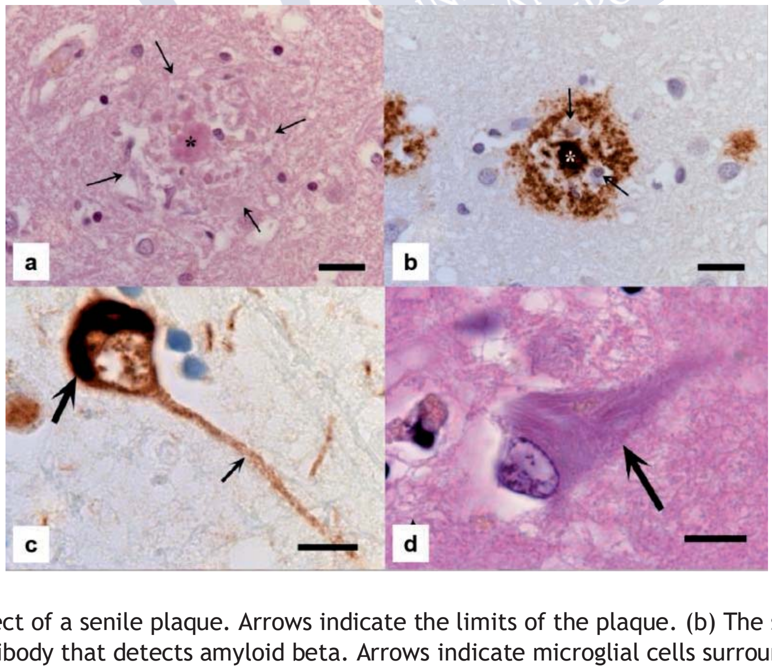

Figure 16. (a) Aspect of a senile plaque. Arrows indicate the limits of the plaque. (b) The same senile plaque stained with an antibody that detects amyloid beta. Arrows indicate microglial cells surrounding the plaque (c) Neuron with tau aggregates, arrows indicate tau in the body and the apical dendrite of the cell (d) Neurofibrillary tangle in the cell body of a neuron (indicated by arrow). The tangle is stained in blue. Scale bars: 10 μM (Calderon-Garciduenas and Duyckaerts, 2017)

Particularly, neurons that use acetylcholine or glutamate as neurotransmitters are affected, but also neurons that produce serotonin or norepinephrine. Neuronal loss occurs as a late event and can reach a 90% in some regions such as the entorhinal cortex. Macroscopically, the hippocampus, the entorhinal cortex and the amygdala are atrophied. In fact, there is a reduction in the brain volume that can reach a 10% per year in those regions (Calderon-Garciduenas and Duyckaerts, 2017; Jahn, 2013; Mattson, 2004).

There is a complex network of molecular and cellular abnormalities that occurs in AD brains and leads to the synaptic dysfunction and cell death observed in the illness. Senile plaques and NFTs are produced by the aberrant accumulation of two proteins, amyloid beta (Aβ) and tau. Degenerated neurons also exhibit increased oxidative damage, impaired energy metabolism, perturbed calcium homeostasis and mitochondrial dysfunction (Mattson, 2004). Many of these molecular alterations occur during normal ageing, but are exacerbated in neurodegenerative diseases (Mattson and Magnus, 2006). Moreover, augmented levels of neuroinflammatory markers and activated glial cells have been detected surrounding the senile plaques (Querfurth and LaFerla, 2010). Although the exact pathological basis of the disease remains unknown, in the following sections the main molecular and cellular mechanisms involved in the disease will be discussed.

1.3.1.1. Oxidative stress

Molecular oxygen is essential for life of aerobic organisms. The use of oxygen provides advantages over anaerobic organisms, as much more energy can be generated, but it also has a cost (Ortiz et al., 2017). Oxygen has a high redox potential, being capable of accepting electrons from reduced substrates and it is kinetically stable, but in presence of some molecules the molecule can be partially reduced, resulting in very unstable and active forms, called reactive oxygen species (ROS) (Gandhi and Abramov, 2012). In particular, the mitochondrial production of adenosine triphosphate (ATP) through oxidative phosphorylation is the most important source of ROS. Under physiological conditions, ROS emission only represents a 2% of the total oxygen consumed, but in pathological circumstances the release of these harmful molecules augments. This increase in ROS levels, along with a reduction in the ability of antioxidant systems to neutralize these molecules, results in oxidative stress (Figure 17) (Zorov et al., 2014).

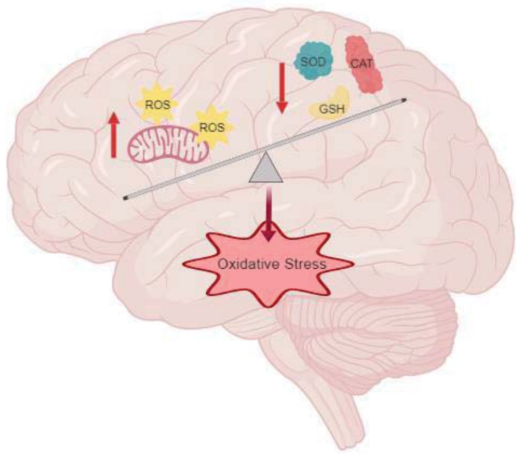

Figure 17. Oxidative stress in neurodegeneration

Oxidative stress plays a crucial role in age-related illnesses such as arthritis, diabetes, cancer, atherosclerosis and neurodegenerative diseases, since ROS accumulation damages many cellular components such as proteins, lipids and nucleic acids, and can even lead to cell death (Tan et al., 2018).

The brain is very susceptible to oxidative damage, it only constitutes the 2% of body weight, but it consumes about 20% of the total oxygen supply. Moreover, the high content of unsaturated fatty acids and relative low levels of antioxidant defences in comparison to other organs contribute to its sensitivity to oxidative injury (Nunomura et al., 2006). There are several evidences of oxidative stress in AD patients. Lipid, protein, DNA oxidation markers and changes in antioxidant systems expression and activity have been detected both in the central nervous system (CNS) and in peripheral tissues (Chen and Zhong, 2014). Moreover, data suggest that oxidative stress is an early event in the illness that precedes Aβ aggregation (Cadonic et al., 2016).

- Reactive oxygen species

The term ROS includes both oxygen radicals, such as superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$), and non-radicals that are easily converted into free radicals, such as hydrogen peroxide (H_2O_2). ROS can be interconverted from one to another by enzymatic and non-enzymatic mechanisms.

The superoxide anion is the most abundant and the precursor of most ROS. H_2O_2 can be produced from $O_2^{\cdot-}$ by both spontaneous and catalysed reactions. Otherwise, the hydroxyl radical is the most potent specie and the main responsible of DNA damage. It can be produced from $O_2^{\cdot-}$ and H_2O_2 in the presence of iron ions (Zorov et al., 2014).

Despite the deleterious effects of ROS accumulation, when their concentrations are kept at low levels, these molecules exert important roles in physiological processes and body defences, acting as signalling molecules. Processes such as cell survival, differentiation, or inflammation are tightly regulated by ROS. For example, increased levels of H_2O_2 are known to induce autophagic cell death (Sena and Chandel, 2012), and the mitogen-activated protein kinases (MAPKs), enzymes involved in cell death and inflammation, are activated by ROS (Li et al., 2013). Therefore, when the levels of these molecules are outside of their normal range, either under conditions of oxidative stress or reductive stress (diminished ROS release), the instability of the redox environment can be harmful if it is not compensated (Zorov et al., 2014).

- Antioxidant defences

A biological antioxidant is any compound that, when present at lower concentrations compared to an oxidizable substrate, is able to delay or prevent the oxidation of that molecule. Among their functions, antioxidants protect from oxidative damage, DNA mutations and other markers of cell damage. However, at sustained ROS release, the antioxidant capacity can be exceeded, resulting in oxidative stress and disease occurrence (Pisoschi and Pop, 2015).

The protective antioxidant mechanisms of cells can be classified into enzymatic and non-enzymatic systems. The major antioxidant enzymes are described below (Figure 18):

- Superoxide dismutases (SODs): this family is specialized in eliminating superoxide ions generated as by-products of oxygen metabolism through the electron transport chain (ETC). There are three isoforms of SOD: Cu/ZnSOD, also called SOD1;

MnSOD or SOD2 and extracellular SOD or SOD3. These forms exert similar functions, but their cellular localization differs. SOD1 is in the cytoplasm, SOD2 is localized in the mitochondria and SOD3 isoform is secreted into the extracellular space.

- Catalase (CAT): is responsible of the conversion of H_2O_2 to water. It is localised in the peroxisomes, the cytosol and the mitochondria. This enzyme has a minor role when H_2O_2 levels are low, but it becomes important when the amount of this molecule augments (Gandhi and Abramov, 2012).
- Glutathione peroxidases (GPxs): are a family of enzymes that catalyse the reduction of H_2O_2 and organic hydroperoxides to water or corresponding alcohols by using glutathione (GSH) as electron donor. There are four isoforms, highly conserved, found in cytosol and mitochondria (Lubos et al., 2011).

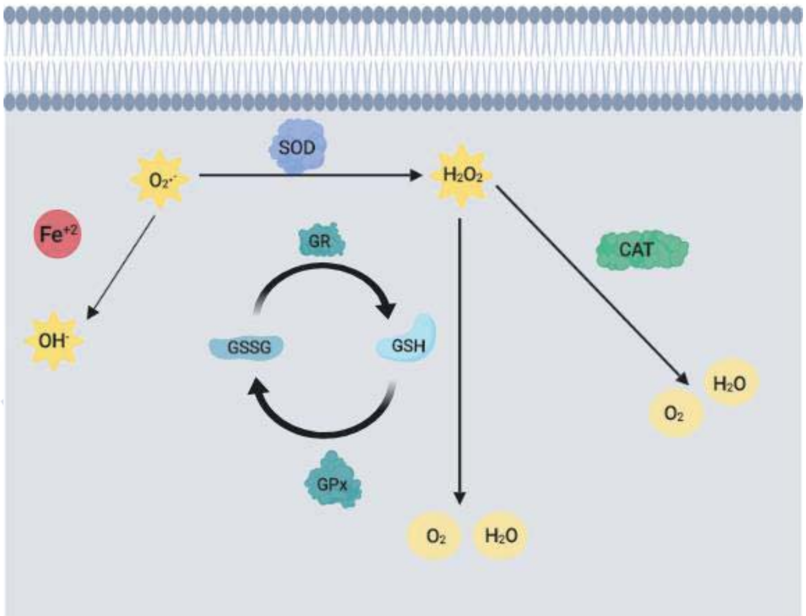

Figure 18. Main antioxidant systems in cells

The non-enzymatic systems are molecules that directly react with ROS and reduce them. The most important are GSH and vitamin E (VitE):

- GSH: is the main antioxidant in the CNS, the most abundant small molecule, present in millimolar concentrations in neurons (Dringen and Hirrlinger, 2003). GSH is a thiol-containing molecule, capable of reacting with ROS and lipid peroxidation products. After the reaction with free radicals, glutathione disulfide (GSSG) is formed. This process can be catalysed by the enzyme GPx or occur independently. Then, GSSG is reduced back by the glutathione reductase (GR) to produce two molecules of GSH, utilizing nicotinamide adenine dinucleotide phosphate (NADPH) as electron donor. GSH levels are reduced in AD patients, whereas GSSG levels are increased. GSSG/GSH ratio is used as a marker of redox status, and its levels increase with the progression of the illness (Pocernich and Butterfield, 2012).

- VitE: or α-tocopherol, is a lipophilic molecule located in cell membranes. VitE maintains membrane function by acting as an efficient scavenger of lipid peroxidation products (Pisoschi and Pop, 2015). Animals obtain VitE by food intake, and its deficiency leads to pathologies such as ataxia or neurological disorders (Traber and Atkinson, 2007).

- Nrf2 pathway

The nuclear factor E2-related factor 2 (Nrf2) is a transcription factor responsible of the induction of a variety of antioxidant and detoxifying genes. Under basal conditions, Nrf2 is degraded by the kelch-like ECH-associated protein 1 (Keap1)-mediated ubiquitin proteasome system. Keap1 acts as an adaptor for the Cullin3 -based ubiquitin E3 ligase complex and retains Nrf2 in the cytosol, preventing its translocation to the nucleus (Figure 19) (Magesh et al., 2012).

Keap1 contains reactive cysteine residues, which are sensors of electrophilic and oxidative signals. Upon exposure to cellular stresses (e.g. oxidative, endoplasmic reticulum stress) or to small molecules called inducers (both endogenous and exogenous), Keap1 residues are modified, preventing Nrf2 degradation and allowing its translocation to the nucleus. Once in the nucleus, the transcription factor binds with small Maf proteins and regulates the expression of antioxidant response element (ARE) genes. The ARE is an enhancer element located in the 5' region of several genes involved in antioxidant systems. Enzymes like SODs, CAT, thioredoxin, sulforedoxin and proteins of the GSH cycle are regulated by Nrf2 (Gan and Johnson, 2014). Nrf2 also improves mitochondrial function, it increases the mitochondrial membrane potential ($\Delta\Psi_m$), the availability of substrates for respiration, and ATP production by enhancing the expression on genes involved in the glucose metabolism (glycolysis, gluconeogenesis, tricarboxylic acid cycle) (Holmstrom et al., 2016). Other genes regulated by Nrf2 include those associated with autophagy and proteasome activation (Esteras et al., 2016).

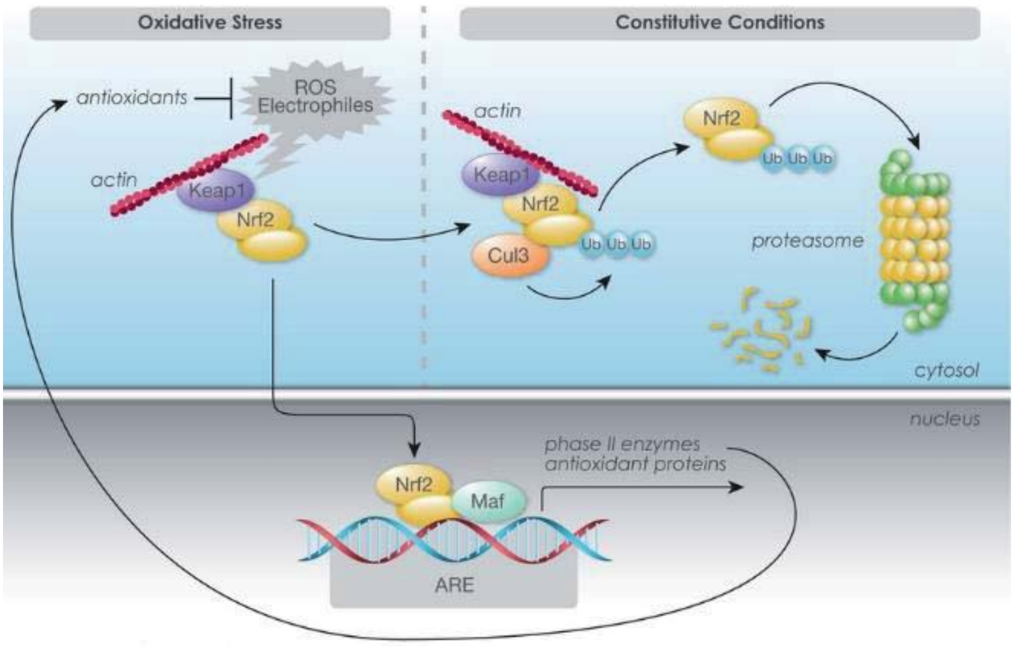

Figure 19. Nrf2 pathway under oxidative and basal conditions (May, 2012)

The protective characteristics of the transcription factor have driven the use of Nrf2 activators as a good therapeutic approach for the treatment of neurodegeneration. AD patients present low levels of Nrf2 activity in the nucleus of hippocampal neurons and downregulation of ARE genes (Dinkova-Kostova et al., 2018), which supports the suitability of the transcription factor as a target for the disease.

1.3.1.2. Mitochondrial dysfunction

Oxidative stress is closely related to mitochondrial dysfunction, since this organelle is the main producer of cellular ROS through the oxidative phosphorylation. Mitochondria have an outer membrane, permeable to small molecules and ions, and an inner membrane, impermeable to most molecules. The inner membrane separates the organelles in two sub-spaces: the mitochondrial matrix and the inter-membrane space.

The ETC takes place in the inner membrane (Figure 20). Four protein complexes comprise the chain: complex I or nicotinamide adenine dinucleotide (NADH)-coenzyme Q oxidoreductase, complex II or succinate-coenzyme Q oxidoreductase, complex III or coenzyme Q-cytochrome c oxidoreductase and complex IV or cytochrome C oxidase. Finally, the ATP synthase (complex V) is also involved in oxidative phosphorylation. The electron flux passes through the complexes until the final acceptor, the oxygen, and generates energy that is used to transport protons across the mitochondrial inner membrane, creating a gradient that favours the flow back through the ATP synthase. However, some electrons leak out the ETC and generate superoxide anion, which represents the 90% of endogenous ROS (Cadonic et al., 2016).

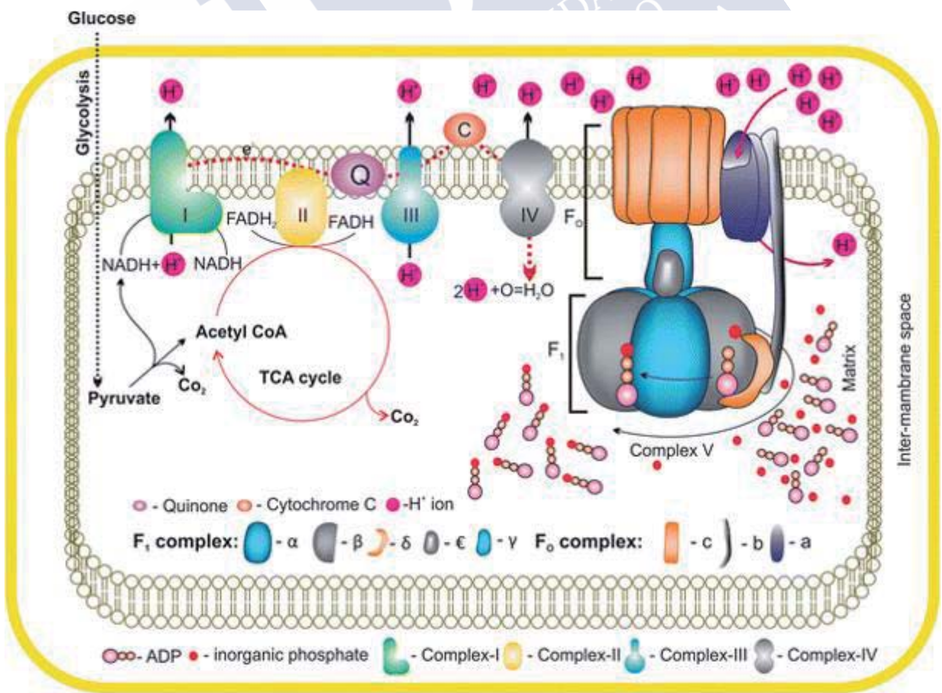

Figure 20. Scheme of oxidative phosphorylation (Cadonic et al., 2016)

Mitochondria, as the main source of ROS, are also the main target of oxidative damage. The progressive impairment of mitochondrial function has been involved in aging and neurodegeneration. Oxidative stress and mitochondrial dysfunction create a vicious cycle that plays an important role in the pathogenesis of AD (Wang et al., 2014). Mitochondria from AD patients present reduced energy metabolism, alterations in enzymes of ETC, mutations in their DNA and abnormal dynamics (Cadonic et al., 2016; Cardoso et al., 2017).

- Mitochondria and cell death

Mitochondria play a central role in the regulation of several forms of cell death, including apoptosis and necrosis. Apoptosis is a programmed cell death, fundamental for maintenance of tissue homeostasis. Two types of apoptosis have been identified: the intrinsic and extrinsic pathways (Figure 21). Both types are characterized by the activation of a group of cysteine proteases, the caspases. There are two kinds of caspases, initiator caspases (caspases 8 and 9) and executioner caspases (caspases 3, 6 and 7). The extrinsic pathway is initiated by binding of cell ligands to death receptors in the cell membrane, inducing the activation of caspase 8. This enzyme stimulates the downstream caspase cascade, including caspase 3 that targets several apoptotic substrates, and leads to apoptotic cell death. In the intrinsic pathway, some signals such as calcium overload, DNA damage or oxidative stress, produce the opening of the mPTP and the collapse of $\Delta\Psi_m$. Then, apoptotic factors, such as cytochrome C, are released from mitochondria and activate caspase 9, which initiates the apoptotic cascade through caspase 3 stimulation (Vakifahmetoglu-Norberg et al., 2017). Apoptotic cell death produces cells clearance, with minimal damage to surrounding tissues.

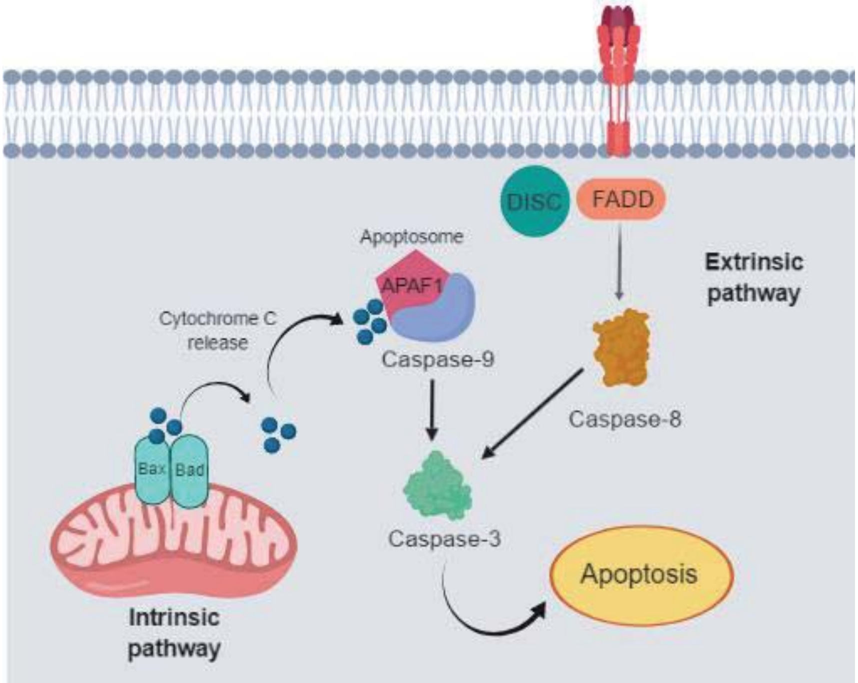

Figure 21. Intrinsic and extrinsic apoptotic pathways

On the other side, necrosis, an uncontrolled cell death process, produces the spillage of cell content and the subsequent damage to surrounding tissues. Necrosis is induced by an external injury, such as hypoxia or inflammation. Necrotic cells are characterized by mitochondrial swelling, loss of $\Delta\Psi_m$ and impaired ATP generation. It is an energy-independent form of cell death (D'Arcy, 2019).

- Mitochondrial permeability transition pore

The mPTP is a large non-specific channel that connects the mitochondrial matrix and the cytosol. The pore is voltage-dependent, activated by matrix calcium overload and ROS and Aβ accumulation. Particularly, ROS sensitize mPTP opening by decreasing the calcium concentration needed to initiate the pore opening.

The channel allows the passage of solutes with a molecular weight less than 1500 Da, provoking an increase in the permeability of the inner mitochondrial membrane. mPTP opening is reversible, providing a pathway to release ROS and calcium from mitochondria, but if it is massive and persistent, it can produce mitochondrial swelling and rupture (Panel et al., 2018). In that case, the pore causes a sudden collapse of the proton gradient, the failure of the ETC and a decrease in ATP production (Rao et al., 2014). These alterations finally lead to cell death through apoptosis or necrosis. If ATP levels become very compromised, mPTP causes necrotic death, nevertheless, in presence of sufficient amount of ATP, the apoptotic process will be triggered (Kalani et al., 2018).

The nature of mPTP remains mostly unknown, its definitive biomolecular composition has not been determined. Traditionally, it was thought that the pore was formed by the adenine nucleotide translocase (ANT) in the inner membrane and the voltage-dependent anion channel (VDAC) in the outer membrane, but when these proteins are deleted genetically, mPTP opening still happens (Briston et al., 2019). Other proteins have been proposed as pore-forming molecules: in the inner membrane, the mitochondrial phosphate carrier (PiC) or the metalloprotease spastic paraplegia7; in the outer membrane, BAK/BAX and the translocator protein. Studies trying to test these hypotheses have failed, but some of these molecules are known to play a regulatory role in mPTP opening. For example, ANT is considered a modulator, and the pore opens less easily in absence of the PiC. Therefore, these proteins contribute but are not indispensable to mPTP generation (Biasutto et al., 2016; Briston et al., 2019). Recent studies have purposed the ATP-synthase as a member of the mitochondrial channel in the outer membrane, but there are opposite results (Antoniel et al., 2018; Biasutto et al., 2016). Anyway, this protein also seems to be involved in the formation of mPTP (Figure 22).

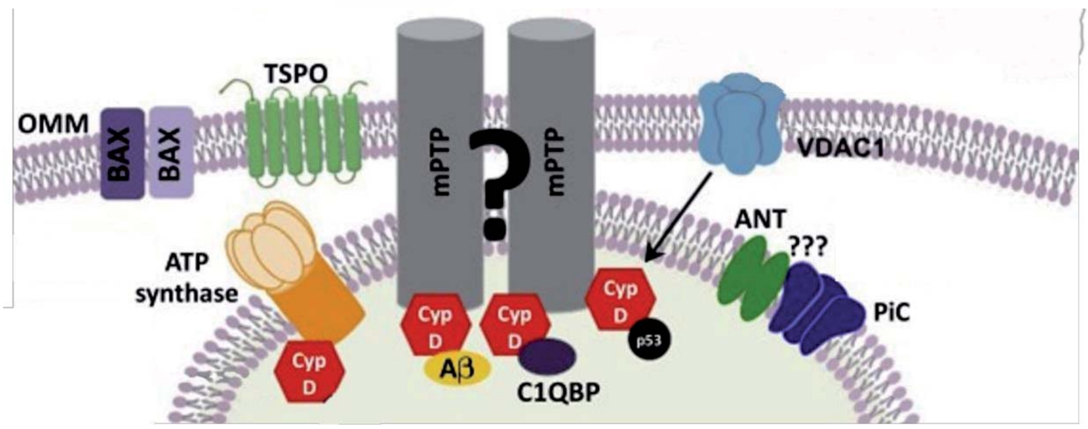

Figure 22. Mitochondrial permeability transition pore (mPTP). Modified from (Briston et al., 2019)

The only molecule that has been confirmed as a component of the mitochondrial pore is CypD. This protein belongs to the cyclophilins family, a highly conserved group of peptidyl-prolyl isomerases (PPIase's) that catalyse the conversion of molecules from *cis* to *trans* conformation and *vice versa*. In humans, there are 18 isoforms located in different cellular compartments: e.g. CypA in the cytosol, cyclophilins B and C mainly in the endoplasmic reticulum, and CypD is the only mitochondrial isoform (Perrucci et al., 2015). The enzymes present two conserved pockets that constitute their active site. In case of CypD, it has a "backface" in the opposite side of the pockets that is thought to mediate protein- protein interactions and shows a less conserved structure than the catalytic core compared to the cytosolic CypA (Gutierrez-Aguilar and Baines, 2015).

CypD is the main regulator of mPTP opening. The enzyme is known to interact with the putative members of the pore ANT, ATP-synthase and PiC, triggering its formation (Biasutto et al., 2016). Many proteins such as p53, the complement C1Q binding protein, Aβ and mitochondrial chaperones are modulators of CypD, becoming mPTP regulators too (Briston et al., 2019).

In AD, ROS and Aβ accumulation enhances mPTP opening. Furthermore, increased levels of CypD have been detected in AD brains, and the genetic deletion of the protein results in memory improvement and attenuated neuronal dysfunction in a mouse model (Du et al., 2008; Du and Yan, 2010). Therefore, the implication of mPTP and, more particularly, of CypD in the disease is outstanding.

1.3.1.3. Neuroinflammation

Inflammation is a physiological response against several stimuli like infection, trauma or disease. Immune cells are recruited to the damaged area through pro-inflammatory signalling, and different responses begin, such as increased vascularization or initiation of pathogen phagocytosis. Once the injury is neutralized, immune cells change their phenotype via anti-inflammatory signals. In this phenotypic state, the release of pro-inflammatory factors decreases and the anti-inflammatory mediators augment, producing the repair of the tissue and the clearance of debris (Newcombe et al., 2018).

Several inflammatory markers have been detected in AD brains, providing evidences that the pathogenesis not only affects to neurons, the immune system is also implicated. The sustained presence of Aβ, hyperphosphorylated tau and ROS results in the activation of cells involved in inflammation. At the beginning of the disease, immune cells try to eliminate protein aggregates and damaged neurons, but the large and sustained exposure to toxic molecules produces a chronic inflammatory response characterized by high pro-inflammatory factors release, which can finally lead to neuronal death (Clayton et al., 2017).

- Cellular components of neuroinflammation

The main cellular mediators of inflammatory processes in the brain are astrocytes, monocytes, macrophages and microglia (Figure 23). Surprisingly, neurons also contribute to neuroinflammation by releasing inflammatory molecules.

Astrocytes are the most abundant cells in the CNS. They are involved in the connective tissue and skeletal function of the brain, supporting endothelial cells from the blood brain barrier (BBB). These cells also participate in the maintenance of neuronal synapses, ion balance, nutrient supply and Aβ clearance. Under inflammatory conditions, astrocytes release cytokines and other toxic molecules, exacerbating the neuroinflammatory response. Besides, astrocyte dysfunction can damage synaptic homeostasis, initiating the neuronal injury (Bagyinszky et al., 2017; Tuppo and Arias, 2005).

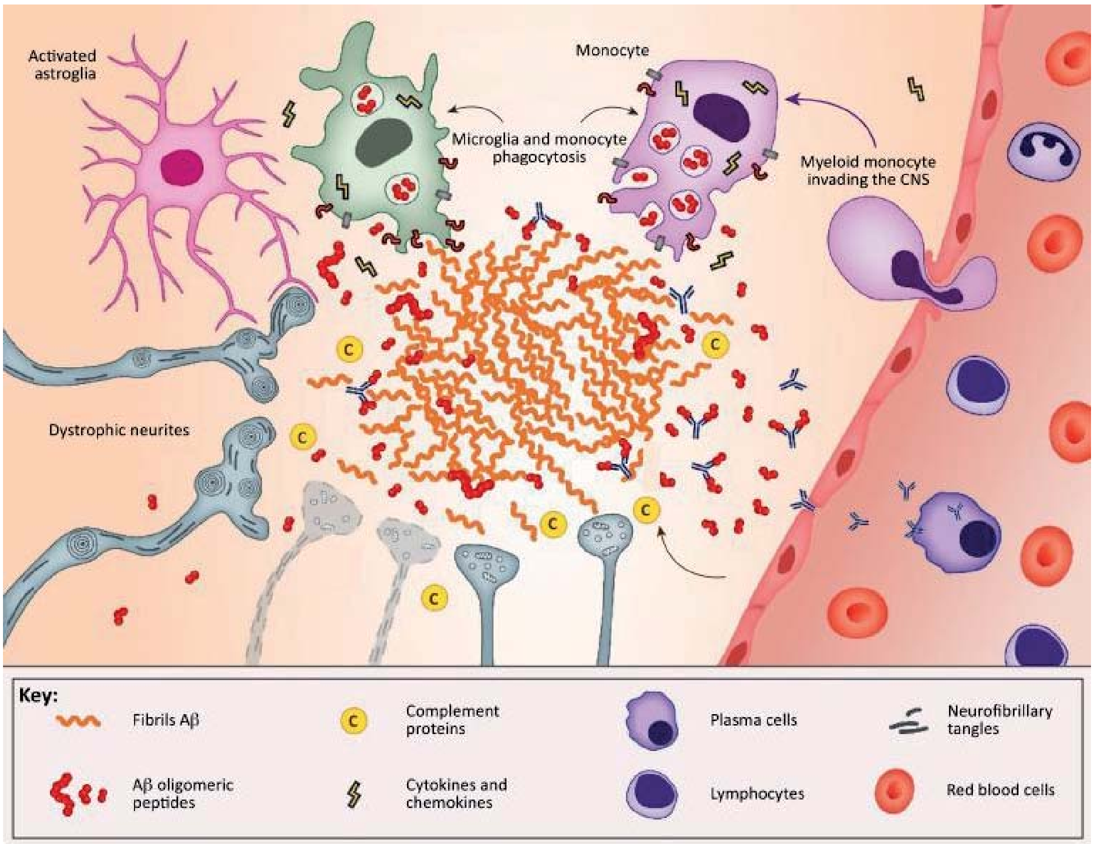

Figure 23. Cells involved in the neuroinflammatory response in AD. Modified from (Cuello, 2017)

Peripheral blood monocytes differentiate into macrophages in response to injury. Macrophages can cross the BBB and infiltrate the CNS, where they participate in the clearance of amyloid deposits. However, if there is an inflammatory surrounding environment, macrophages will produce pro-inflammatory mediators. The contribution of these cells to AD is controversial, but the disruption of BBB integrity with age and results from animal models suggest that infiltrating macrophages are also implicated in the illness (Bagyinszky et al., 2017; Minogue, 2017).

Microglia are the main responsible of immune response in the brain. Microglial cells account around 10-15% of the glia and participate in the maintenance of synapses, neurogenesis and regulation of cognitive functions. Microglia are involved in the phagocytosis of cell debris generated by apoptotic neurons, a crucial function for the maintenance of brain homeostasis (ElAli and Rivest, 2016; Li et al., 2018b). Morphologically, resting microglia present a small soma and ramifications, but upon activation the cells acquire an amoeboid aspect by enlarging their soma and shortening their processes (Newcombe et al., 2018). Under pathological situations, microglia migrate to surround damaged cells and respond with phagocytosis and by releasing inflammatory mediators (cytokines, chemokines, ROS…).

Neurons, which were traditionally thought to be passive participants in inflammation, may also produce inflammatory molecules and serve as a source of complement system, but their contribution is smaller (Bagyinszky et al., 2017).

- Cytokines and chemokines

The neuroinflammatory response observed in AD is characterized by the presence of pro- and anti-inflammatory mediators like cytokines or chemokines, and the activation or downregulation of inflammatory enzymatic systems. The combined expression of all these molecules and pathways contributes to neurodegeneration.

Cytokines are a group of small polypeptides that act at low concentrations (picomolar to nanomolar) and can modulate cellular processes like cell survival or differentiation. Upon infection or disease, cytokines are upregulated and play an important function in tissue repair. Cytokines are classified in pro- and anti-inflammatory molecules (Smith et al., 2012):

- Pro-inflammatory cytokines: these proteins are released in inflammatory processes to regulate the intensity and duration of the immune response. The most important pro-inflammatory cytokines in AD are interleukin 1-beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis-alpha (TNF-α). These molecules impair neuronal function and structure, and are upregulated in brains and peripheral tissues from AD patients (Heneka et al., 2015). IL-1β is an important activator of inflammation and produces neuronal degeneration. This cytokine enhances the production of IL-6, which also generates astrogliosis and microgliosis and stimulates the MAPKs cascade. TNF-α is a crucial mediator in the initiation and regulation of the immune response, the cytokine is generated by neurons and microglia and activates these cells, triggering their own production. It has also been reported that TNF-α can induce the stimulation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), a key regulator in the immune response (Fischer and Maier, 2015). There are other pro-inflammatory cytokines involved in neurodegeneration such as interferon-γ, granulocyte-macrophage stimulating factor (GM-CSF) or interleukin-18 (Domingues et al., 2017; Heneka et al., 2015).
- Anti-inflammatory cytokines: these molecules suppress the production and activity of pro-inflammatory cytokines. For example, interleukin-4 (IL-4) has neuroprotective effects through the inhibition of TNF-α and NO levels, interleukin-10 (IL-10) reduces the synthesis of IL-1β and TNF-α by decreasing the expression of their receptor, and transforming growth factor beta (TGF-β) also has anti-inflammatory properties by modulating Aβ accumulation and regulating cell death (Rubio-Perez and Morillas-Ruiz, 2012).
- Chemokines: are a family of cytokines that present chemotactic properties. There are over 50 different molecules in this group that regulate immune cells migration and recruitment to the inflammatory area (Domingues et al., 2017). In AD, increased levels of CCL2, CCR3 and CCR5 have been detected and Aβ stimulates the production of interleukin-8, MCP-1 and MIP-1α (Akiyama et al., 2000; Heneka et al., 2015).

Hence, cytokines and chemokines are crucial players in the neurodegenerative disorder, acting as promoters or suppressors at different cellular levels such as Aβ processing or tau phosphorylation (Domingues et al., 2017).

- Nitric oxide

NO is synthetized by the nitric oxide synthases (NOS) through the conversion of L-arginine to NO and L-citrulline. There are three isoforms of the enzyme: NOS1 or neuronal NOS (nNOS), NOS2 or inducible NOS (iNOS) and NOS3 or endothelial NOS (eNOS). In the brain, nNOS is expressed in the synaptic spines, astrocytes and the connective tissue that surround blood vessels, iNOS is present in astrocytes and microglia, and eNOS in vascular endothelial cells and motor neurons. eNOS and nNOS are constitutively expressed and their activity is calcium-dependent, whereas iNOS is calcium-independent and its activity is induced during inflammatory events (Balez and Ooi, 2016; Yuste et al., 2015).

NO can freely diffuse across membranes and has physiological roles such as vasodilator, neuromodulator and inflammatory mediator. The molecule also participates in S-nitrosylation of proteins, which is associated with muscular contraction and apoptosis. Upon activation, glial cells highly express iNOS, resulting in augmented levels of the molecule. When NO is produced in large quantities, it becomes neurotoxic and can affect to mitochondrial respiration, CAT activity or enhance protein nitration (Yuste et al., 2015). NO can also react with ROS, resulting in the formation of peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2) and nitrogen trioxide (N_2O_3). These molecules are named reactive nitrogen species (RNS) and cause a non-selective repression of mitochondrial components, stimulating ROS generation, oxidative damage and producing neuronal death (Brown, 2010).

The chronic induction of the immune response also contributes to the oxidative environment observed in AD. Along with ROS release induced by NO, microglia produces elevated levels of these damaging molecules by the NADPH oxidase (NOX). The enzyme is assembled and activated upon stimulation of the cells, and predominantly generates extracellular superoxide (Brown and Vilalta, 2015). NOX2 is the prevalent isoform in microglial cells, located in the plasma membrane. The enzyme generates superoxide by transferring electrons to molecular oxygen on one side of the membrane, while oxidizing NADPH to $NADP^+$ and H^+ on the other side. Importantly, activated microglia presented a decrease in O_2 consumption and ATP production, and increased glycolytic activity. Glucose fuels the production of ROS, as the rapid superoxide production by the NOX requires the continuous supply of NADPH through the glucose metabolism. Moreover, glucose is the origin of reducing equivalents that enter the ETC and can enhance the production of ROS in the mitochondria. However, mitochondrial ROS contribution to the inflammatory cascade is much lesser than the superoxide generation by NOX (Ghosh et al., 2018).

- Pathways involved in neuroinflammation

The description of the principal pathways implicated in the inflammatory cascade is focused on microglia, as they are the main cellular mediators of neuroinflammation. The presence of an initiating insult like Aβ or ROS produces the activation of the cells through the toll-like receptors (TLRs), located in the plasmatic membrane. The binding to TLRs triggers several transduction pathways as MAPKs and NFkB (Shabab et al., 2017).

NFkB is a transcription factor composed by five domains: p50, p52, p65, RelB and c-Rel. These proteins form homo- and hetero- dimers with different affinity towards specific sequences of DNA. Dimers remain in an inactive form in complex with the IkB proteins. The canonical activation of NFkB involves the stimulation of the IkB kinase (IKK) complex, composed by IKKα, IKKβ and IKKγ. Activation of IKK produces the phosphorylation of IkB proteins by IKKβ. IkB are degraded in the proteasome, producing the release of NFkB dimers and their translocation to the nucleus, where they bind to specific DNA targets (Figure 24). Some genes regulated by NFkB encode cytokines, chemokines, immunoreceptors, and regulators of apoptosis. Particularly, the p65/p50 dimer is an inducer of pro-inflammatory genes. The increase of p65/p50 activation leads to augmented levels of iNOS expression and release of ROS and pro-inflammatory cytokines such as IL-1β or IL-6. Besides, these neurotoxic mediators enhance NFkB, generating a vicious cycle that precipitates neurodegeneration (Srinivasan and Lahiri, 2015).

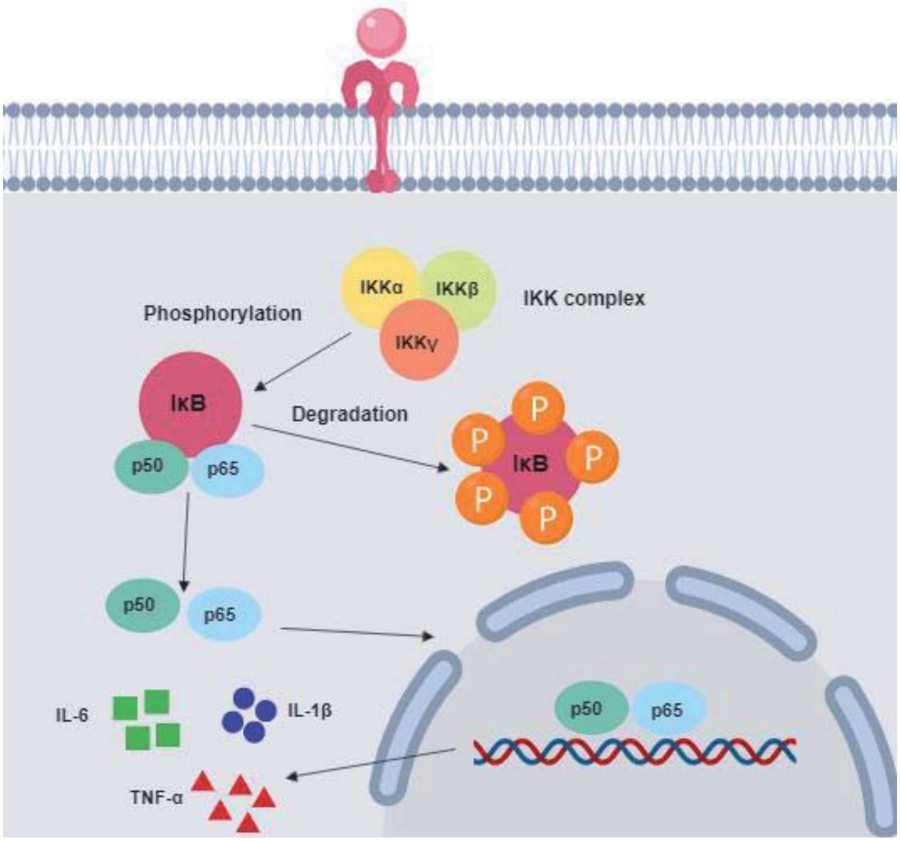

Figure 24.Canonical activation of NFkB

Microglia stimulation also triggers the activation of MAPKs, which include p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). These enzymes are serine-threonine kinases that have important roles in growth, differentiation, cell cycle and cell death. MAPKs are activated in response to stress and inflammation and in turn, stimulate mediators of the inflammatory cascade. The ERK pathway can be activated by growth factors and cytokines like IL-1β and TNF-α (Zhang et al., 2016b). JNK and p38 are also activated by pro-inflammatory cytokines and in response to osmotic, hypoxic or oxidative stress (Kim and Choi, 2015). MAPKs enhance neuroinflammation by upregulating iNOS expression and by increasing cytokine production (Kaminska, 2005).

Nrf2 is also related to the regulation of the innate immune response. The transcription factor represses the activation of pro-inflammatory genes and potentiates the anti-inflammatory signalling. Nrf2 inhibits the transcription of IL-6 and IL-1β by binding to DNA sequences near to their genes (Quinti et al., 2017). Besides, the upregulation of antioxidant genes by Nrf2 contributes to its anti-inflammatory role, since ROS overproduction promotes neuroinflammation (Rojo et al., 2014).

- Microglial polarization

Neuroinflammation is considered a double-edged sword with both beneficial and detrimental effects over the neurons. Traditionally, microglial cells have been considered harmful to neurons due to their ability to secrete toxic molecules, but this view has changed because of the importance of these cells in neuroprotection, trophic factor production and plasticity. Microglia are very ductile cells that can present a great range of phenotypes that fluctuate between two opposite extremes: M1 phenotype and M2 phenotype (Figure 25). The M1/M2 model is a simplified way to explain the broad spectrum of microglial states. The M1 or "classically activated" phenotype is characterized by high levels of ROS and NO release, and by the production of cytokines such as IL-6, IL-1β and TNF-α. The M2 phenotype is also named "alternative activated" state and is associated with anti-inflammatory molecules, such as IL-10 or IL-4, and neurotrophic factors such as TGF-β, which can suppress pro-inflammatory responses. M2 microglia also presents a high phagocytic capacity (Pena-Altamira et al., 2016; Tang and Le, 2016).

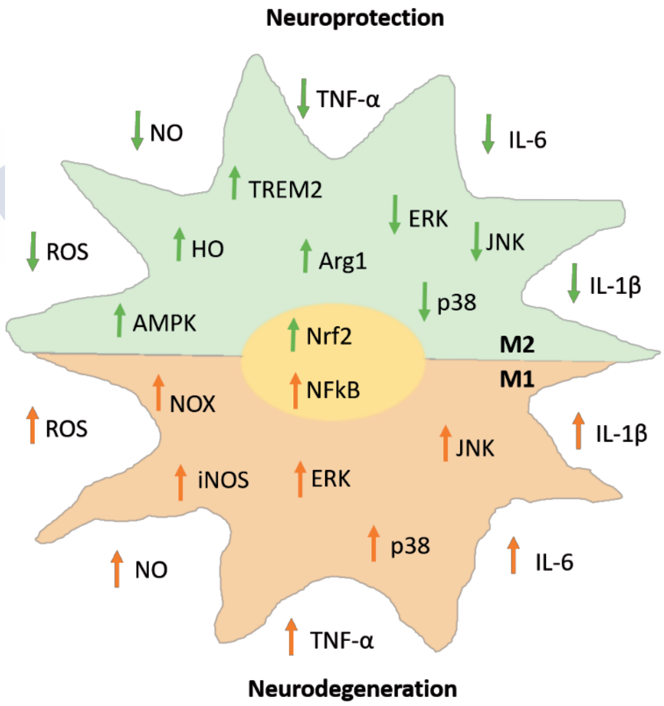

Figure 25. M1 and M2 microglial phenotypes. The upper part represents the main pathways and molecules up- and down-regulated in the neuroprotective phenotype. The lower part shows the most important characteristics of M1 phenotype. Modified from (Pena-Altamira et al., 2016)

Redox status exerts an important role in the acquisition and maintenance of microglial phenotypes. In AD, ROS deregulation leads to a defective inflammatory response, with an enhanced M1 phenotype. The transcription factor NFkB, which is redox-sensitive, is activated, generating pro-inflammatory cytokines. Moreover, microglial cells with M1 phenotype produce high levels of ROS and RNS due to the activation of iNOS and NOX, intensifying NFkB stimulation. On the other hand, Nrf2 translocation leads to the expression of antioxidant and anti-inflammatory genes, characteristics of the M2 phenotype. Nrf2 is also affected by the redox state, but its activation is thought to happen more slowly. In this context, NFkB is considered the main regulator of the pro-inflammatory phenotype and Nrf2 may be the master regulator of the anti-inflammatory phenotype (Rojo et al., 2014).

There are many evidences of a crosstalk between these pathways at molecular level. For example, Keap1 degrades IKKβ through ubiquitination, inhibiting the activity of NFkB. Otherwise, NFkB exerts a negative effect on Nrf2, as p65 domain can increase the expression of Keap1, diminishing Nrf2 translocation(Wardyn et al., 2015).

Along with the activation of Nrf2 and NFkB, there are other specific markers of the opposite phenotypes. M1 cells express high levels of cyclooxygenase-2, iNOS, MAPKs, glycogen synthase-3 beta (GSK3β) and NOX, whereas M2 phenotype is characterized by AMP-activated protein kinase (AMPK) stimulation, arginase-1 (Arg-1) expression and high levels of the triggering receptor expressed on myeloid cells 2 (TREM-2), involved in phagocytosis (Cherry et al., 2014; Pena-Altamira et al., 2017; Pena-Altamira et al., 2016).

1.3.1.4. Tau hyperphosphorylation

Tau is an abundant protein in the CNS, involved in microtubule assembly and stabilization. It is mainly associated with axonal microtubules and to a lesser extent with dendrites, where it is involved with signalling functions (Figure 26a). Hyperphosphorylated tau forms the core of NFTs, a pathological hallmark of AD brains that has been traditionally the main focus of therapeutic approaches for the treatment of the pathology (Mietelska-Porowska et al., 2014).

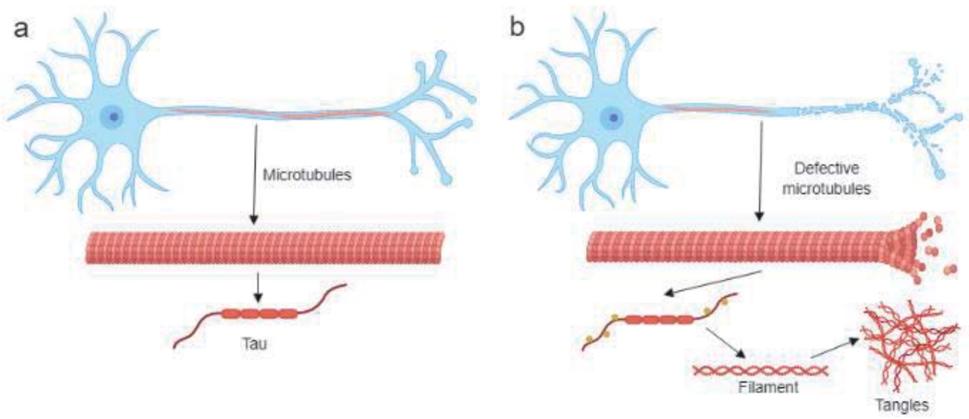

Figure 26. Tau aggregation and neuronal degeneration. (a) In physiological conditions, tau stabilizes microtubules. (b) But when tau becomes hyperphosphorylated, the protein becomes toxic and aggregates

The protein is subject to a complex regulation by post-translational modifications. Tau is modified by phosphorylation, isomerization, glycation, acetylation, oxidation… When post-translational changes are abnormal, tau protein becomes non-functional. Particularly, tau hyperphosphorylation is a key event in AD, responsible of its aggregation. Phosphorylation is a necessary step for the realization of normal physiological functions, but an excessive amount of phosphorylated state reduces the biological activity of proteins. Abnormal phosphorylation converts tau from a functional molecule to a toxic protein (Figure 26b). The loss of microtubule-binding capacity leads to the accumulation of cytosolic tau, increasing tau-tau interactions. The highly flexible structure of the protein allows its interaction with numerous partners, but also with other tau molecules to form oligomers, fibrils and filaments (Jadhav et al., 2019).

- Tau kinases and phosphatases

Tau hyperphosphorylation results from an imbalance between kinases and phosphatases activity. The protein kinases implicated in tau phosphorylation (Figure 27) are divided in three groups: proline-directed protein kinases (PDPKs), non- PDPKs and tyrosine protein kinases (Mietelska-Porowska et al., 2014).

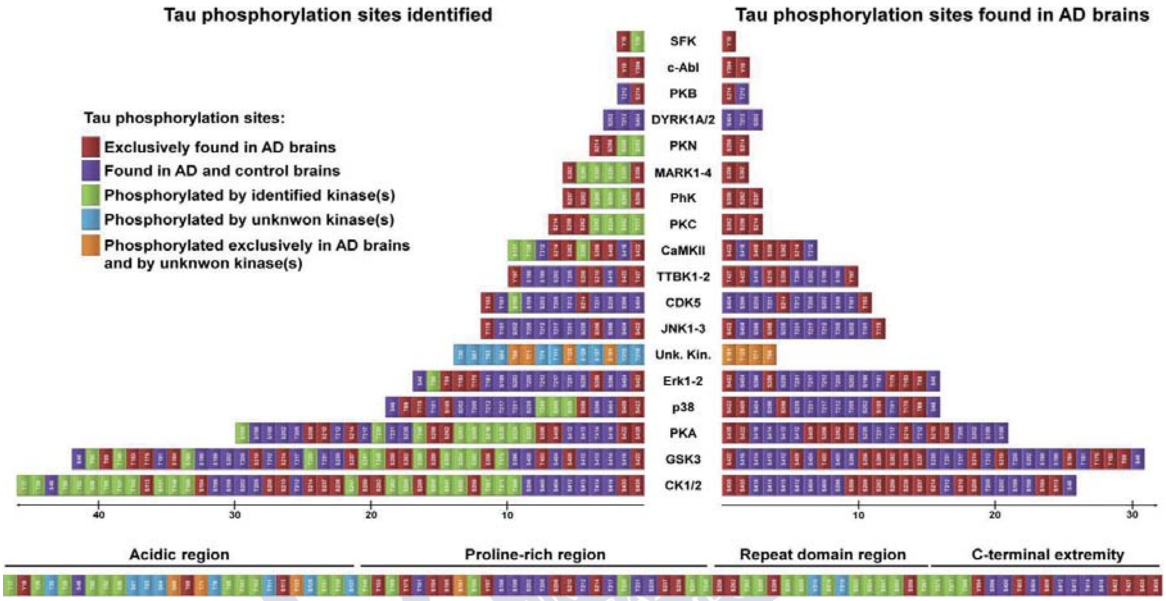

Figure 27. Summary of tau kinases and tau residues phosphorylated in AD(Martin et al., 2013)

The PDPK group includes enzymes such as GSK3β, cyclin-dependent protein kinase-5 (Cdk5) and MAPKs.

- GSK3β is involved in glycogen metabolism, cell proliferation, neuronal functions, apoptosis and the immune response (Maqbool et al., 2016). The kinase is inactivated by phosphorylation at serine 9 and its activation depends on phosphorylation at tyrosine 216. The phosphorylated level of this enzyme is correlated with the progression of neurodegeneration and it has been detected co-localized with NFTs.
- Cdk5 is essential for the development of CNS and the regulation of neuronal cytoskeleton. This kinase is also an upstream regulator of MAPK pathways. The enzyme acts on 11 tau residues, which are found phosphorylated in brains of AD patients. Besides, phosphorylation by Cdk5 promotes the activity of GSK3β (Martin et al., 2013).
- ERK activity has also been associated with abnormal tau phosphorylation in AD. The MAPK phosphorylates tau at 16 sites, 15 of which have been detected in the illness. The enzyme also participates in synaptic plasticity, brain development and repair, and memory formation (Sun and Nan, 2017).
- JNK phosphorylates tau at 12 residues, only identified in neurodegeneration, not in control conditions (Mietelska-Porowska et al., 2014). JNK, as happens with the rest of

tau kinases, regulates several processes such as brain development, repair, inflammation and apoptosis (Mehan et al., 2011).

- p38 is activated by phosphorylation on Thr180 and Tyr182 in response to an extracellular stress or cytokine exposition. The kinase can phosphorylate tau protein at 21 sites. Among them, 15 residues have been detected in AD. Moreover, p38 is able to activate GSK3β, enhancing the protein phosphorylation (Martin et al., 2013).

Among the other classes of tau kinases, it is remarkable the role of the non-PDPK casein kinase 1 (CK1) and the protein kinase A (PKA) (Iqbal et al., 2016). CK1 phosphorylates tau at 46 residues, 25 found in AD brains (Martin et al., 2013), whereas PKA activity becomes the protein a better substrate for GSK3β (Mietelska-Porowska et al., 2014).

Regarding tau phosphatases, the protein phosphatase 2A (PP2A) is the most important, being responsible of about 70% of phosphatase activity in the brain. PP2A can reduce tau phosphorylation directly or by regulating some kinases such as GSK3β or Cdk5 (Iqbal et al., 2016).

1.3.1.5. Aβ pathology

Aβ constitutes, together with NFTs, the main brain alterations observed in AD, and, therefore, has been the other major focus of effort in the search for an effective treatment for this disease. Aβ peptides form senile plaques, which are generated due to an abnormal proteolytic cleavage of APP. This protein is located in the plasmatic membrane, the endoplasmic reticulum or the Golgi complex. It is characterized by a short cytoplasmic region and a large extracellular domain. APP can be processed through two different pathways: non-amyloidogenic and amyloidogenic. There are three enzymes involved in its cleavage: α-, β- and γ-secretases.

The major pathway is the non-amyloidogenic processing, driven by the α-secretase. The enzyme acts between Lys16 and Leu17 of APP, resulting in the formation of a soluble ectodomain, sAPPα, and a membrane-bound C-terminal fragment. This last fragment can be further proteolyzed by γ –secretase to produce small peptides, which are rapidly degraded. The production of sAPPα fragment has been related to an augmented neuronal activity and neurite outgrowth (Figure 28).

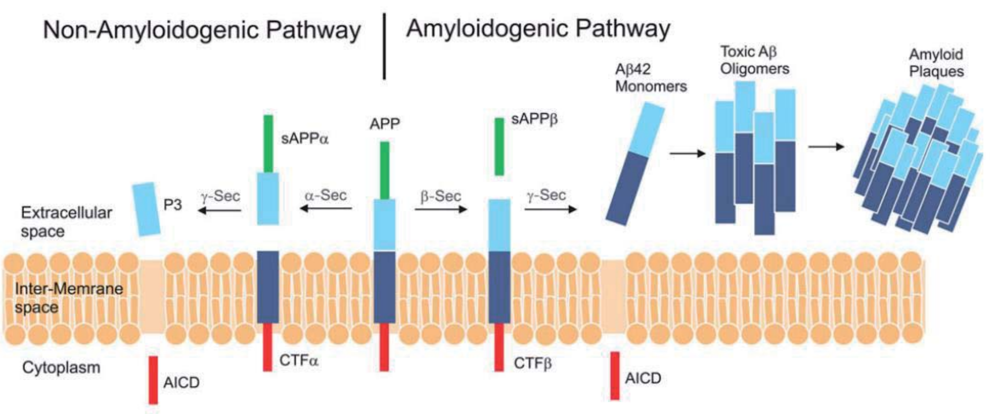

Figure 28. Amyloid precursor protein (APP) cleavage by secretases (Bachurin et al., 2017)

The amyloidogenic pathway involves the sequential cleavage by BACE1 and γ-secretase in the extracellular and cytoplasmic domains, respectively. The proteolysis by BACE1 leads to the formation of the soluble APPβ fragment (sAPPβ) and a membrane-anchored C-terminal fragment (C99). The C99 fragment can be internalized and processed by γ –secretase, releasing Aβ peptides. Aβ fragments length varies from 39 to 43 amino acids, with $A\beta_{1-40}$ and $A\beta_{1-42}$ as the most common peptides. The first one is soluble and less neurotoxic, whereas $A\beta_{1-42}$ is highly neurotoxic, with a greater propensity to aggregate.

Aβ accumulation leads to the formation of soluble dimers, trimers, oligomers, and insoluble fibrils and plaques. Oligomers are the most toxic forms and induce synaptic dysfunction, disruption of calcium homeostasis, and impairments in axonal transport and mitochondrial functions (Huang and Mucke, 2012). Also, as mentioned before, Aβ can activate microglial cells and promote neuroinflammation (Kinney et al., 2018).

At this point, it should be mentioned the inherited form of the pathology, as it is closely related to Aβ accumulation. All the AD-related genetic mutations affect to the processing of this protein. Mutations in APP gene and presenilins, which are part of the active site of γ –secretase, increase Aβ cleavage by the amyloidogenic pathway (Mattson, 2004). For this reason, Aβ accumulation has been traditionally considered as the main cause of AD and several research efforts have been directed to this target (Wang et al., 2017)

1.3.1.6. Autophagy

Autophagy is a regulated process that lead to clearance of damaged proteins and dysfunctional organelles. This cellular machinery is activated by a variety of signals such as nutrient starvation, oxidative stress and neuronal excitotoxicity. Through this pathway, cells can degrade damaged components and restore substrates for energy metabolism (Zare-Shahabadi et al., 2015). Nevertheless, under some circumstances, a sustained or enhanced autophagy activation may lead to cell death (Marino et al., 2014).

Autophagy is an essential pathway in neurons. With age, neuronal cells accumulate damaged organelles, such as mitochondria, which must be eliminated by autophagy to maintain an appropriate homeostasis. Neurons are post-mitotic cells that cannot use mitosis to remove toxic substances and impaired organelles. This characteristic makes neurons more dependent on autophagy for the clearance of proteins and organelles. Many evidences of deregulation of this pathway have been found in AD brains, such as immature autophagic vacuoles and changes in the expression of autophagy-related proteins (Li et al., 2017).

There are three types of autophagy: chaperone-mediated autophagy, microautophagy and macroautophagy. The last type is the major contributor to the autophagic rate, hereafter referred as autophagy, and is characterized by autophagosome formation. Autophagosomes consist in a lipid bilayer that engulf the substrates to be degraded and fuse with the lysosome to form autolysosomes. The process can be divided in three steps: initiation, expansion or elongation and maturation (Zare-Shahabadi et al., 2015).

When the autophagic flux is initiated, the mammalian target of rapamycin (mTOR), considered the key cell growth regulator, is inhibited. This blockage leads to the activation of the Unc-51-like kinase 1 (ULK1) complex, which initiates the autophagic flux by regulating the Beclin1-VPS34 complex (also named class III PI3K complex). The components of this complex are phosphorylated and stimulate the phagophore elongation (Figure 29).

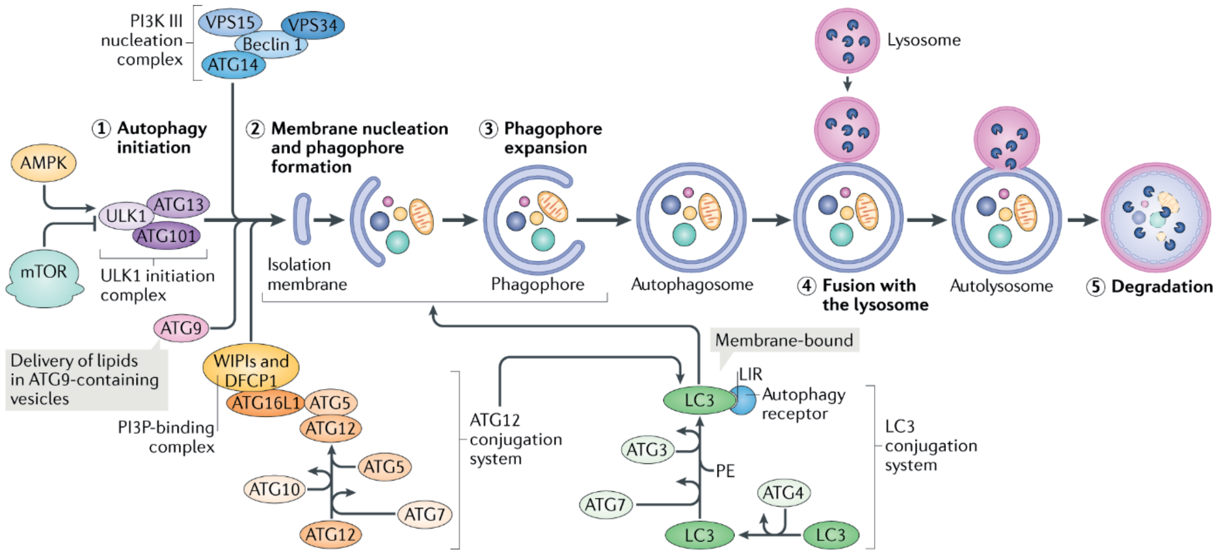

Figure 29. Autophagic flux (Hansen et al., 2018)

The phagophore elongation is controlled by two systems: the ATG5-ATG12 and the microtubule-associated light chain 3 (LC3). The recruitment of membranes from the endoplasmic reticulum, mitochondria or Golgi complex is necessary for the formation of the autophagosome. During this step, LC3I is converted into LC3II, the lipidated form, which is considered the signature of the autophagic membranes. Then, the protein p62, located at autophagosome formation sites, can associate with LC3, acting as the recognition receptor for degradation of proteins and organelles (Liu et al., 2017).

Finally, autophagosome undergoes maturation and fuses with the lysosome, leading to the formation of the autolysosome, with an internal acidic and hydrolytic environment that degrades the damaged cellular components (Dikic and Elazar, 2018; Li et al., 2017).

The role of AMPK in autophagy is also remarkable. The kinase activity is increased when ATP levels are low, leading to the inhibition of mTOR. Moreover, AMPK can directly phosphorylate the ULK1 complex, initiating the autophagic flux (Morgunova and Klebanov, 2019).

1.3.2. Therapeutic strategies

As has been described in the previous sections, AD is a multifactorial illness with a complex aetiology. The precise molecular mechanisms and origin of the pathology remain not fully understood, making the search of therapeutic interventions a great challenge (Bachurin et al., 2017).

Nowadays, there are four approved drugs for the treatment of the disease that offer limited and temporary benefits to patients. All the drugs in clinical practise are focused on the modulation of neurotransmitters, which act maintaining neuronal communication. Three of them are AChE inhibitors: donezepil, galantamine and rivastigmine. These drugs prevent the breakdown of the neurotransmitter acetylcholine produced by the enzyme AChE, enhancing neuronal activity. The other approved drug is memantine, a low-affinity non-competitive antagonist of the *N*-methyl-D-aspartate receptors (NMDAR) that binds to the receptor and reduces glutamate activity, known to produce excitotoxicity and neuronal death (Khan et al., 2017).

However, due to the lack of effectiveness of these drugs to stop or delay the progression of the illness, the drug discovery and development strategy for AD has changed. Several therapeutic approaches based on the main hallmarks of the pathology have been developed, such as those directed towards Aβ and tau accumulation, oxidative stress, mitochondrial dysfunction, neuroinflammation or a multitarget strategy (Table 2). Moreover, the illness is thought to begin many years before the appearance of the first symptoms, so preventive strategies have also been explored. The modulation of risk factors such as diabetes and cardiovascular diseases by changing dietary and exercise habits and cognitive stimulation have also been proposed as therapeutic options (Cao et al., 2018).

Table 2. Summary of pharmacological approaches developed for AD treatment

Pharmacological strategies			
Aβ	Active immunization	Mitochondrial dysfunction	Mitochondrial-targeted antioxidants
	Passive immunization		mPTP inhibitors
	Secretase inhibitors		Nrf2 activators
	Anti-aggregation	Neuroinflammation	Anti-inflammation
Neurotransmitters	Cholinesterase inhibitors		Immunomodulation
	NMDAR antagonists	Others	Autophagy
	Serotonin receptors ligands		Metal chelators
Tau	Anti-aggregation		Glucose metabolism
	Kinase inhibitors		Gene therapy
	Phosphatase activators		Nanotherapeutics
Oxidative stress	Direct antioxidants	Multitarget strategy	
	Indirect antioxidants		

- Drugs affecting Aβ aggregation

The therapeutic strategy focused on Aβ has been the most studied until now, guided by the amyloid cascade hypothesis, which was proposed based on the genetic mutations involved in familial AD. The hypothesis purpose Aβ aggregation as the origin of the illness, which induces tau phosphorylation and the mitochondrial and synaptic dysfunction observed in the disease. This therapeutic approach consists in affecting the stability, removal or aggregation of Aβ. The first clinical strategy was an active immunization trial that started in the year 2000, but some participants developed meningoencephalitis and the trial was ended. Then, passive immunization, which consists in the direct injection of epitope-specific antibodies against Aβ, was tried. Several antibodies targeting monomers, oligomers and/or fibrils have been tested, but none of them displayed significant effects in delaying cognitive impairment. Moreover, some of these drugs produced adverse side effects (Wang et al., 2017). Other approaches with BACE1 and γ-secretase inhibitors or anti-aggregation compounds have been explored and are currently under clinical evaluation (Figure 30) (Bachurin et al., 2017).

However, despite the extensive research focused on Aβ aggregation, none of the drugs has turned out to be effective against the disease (Wang et al., 2017).

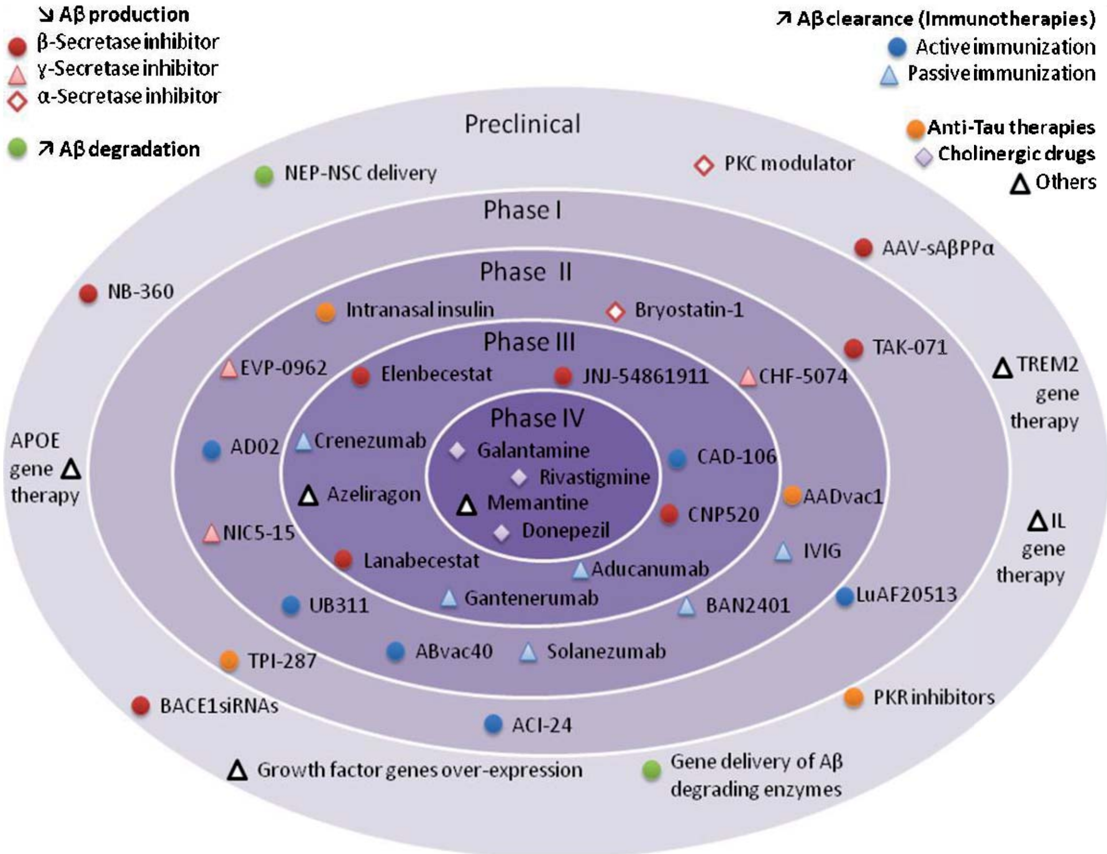

Figure 30. Drugs in development for AD treatment. Therapies are classified by their main target and the most advanced phase of study (Piton et al., 2018)

- Inhibition of tau phosphorylation and aggregation

The tau-based therapeutic approach is the main rival of the anti-amyloid treatments. Due to the failure in Aβ therapies, and based on the observation that NFTs deposition has a strong relationship with the progression of AD, tau therapies have received much attention in recent years (Perez Ortiz and Swerdlow, 2019).

Several approaches to inhibit tau pathology have been exploited: active and passive immunotherapy, tau aggregation inhibitors, phosphatase activators, kinase inhibitors... Tau therapies are more recent than anti-Aβ drugs, so their clinical evaluation is mainly in phase I and II. Only one compound reached phase III, the disinfectant methylene blue. This agent blocked tau polymerization *in vitro* and displayed beneficial effects in transgenic mice. However, the compound produced undesirable side effects, so some derivatives were developed to overcome these problems. These derivatives presented improved safety and entered phase III clinical trials. Nevertheless, although some effects were observed in initial stages, the compounds did not show significant benefits to the patients (Congdon and Sigurdsson, 2018; Jadhav et al., 2019).

Tau immunotherapies have also been developed, there are two vaccines with a safety profile under clinical evaluation, and several antibodies against various tau epitopes have been investigated (Congdon and Sigurdsson, 2018). Other strategies to decrease tau aggregation are focused on modulating the activity of kinases and phosphatases. Most efforts on kinase inhibitors have been directed towards GSK3β. The enzyme inhibitor lithium chloride, used to treat bipolar disorder, has presented positive effects and a phase II clinical trial with the compound in currently ongoing (Tapia-Rojas et al., 2019). Moreover, the upregulation of PP2A activity has been tried as therapeutic option. In fact, the drug memantine, in clinical use for AD as NMDAR receptor antagonist, also enhances the PP2A activity. However, as explained before, it only improved AD symptoms temporally and do not block disease progression. Other phosphatase inhibitor, sodium selenite, was tested in a clinical trial in patients with mild to moderate AD, but no effects on tau levels or cognition were detected (Congdon and Sigurdsson, 2018).

- Targeting oxidative stress and mitochondrial dysfunction

To date, treatment strategies targeting mitochondrial dysfunction have been focused on oxidative stress and apoptosis mitigation. Clinical trials with antioxidants have not shown clear results, and some have had detrimental effects, i.e., vitE presented initial promising results, reducing cognitive decline, but these findings were not reproducible in larger groups (Perez Ortiz and Swerdlow, 2019). Data obtained with direct antioxidants suggests that radical scavenging activity is not enough to produce clinical benefits (Stockburger et al., 2018).

Mitochondrial-targeted antioxidants have shown potential beneficial effects in preclinical studies. Currently, mitoquinone mesylate (MitoQ), a mitochondrial-targeted antioxidant, is being tested in a clinical trial. This compound reduced Aβ accumulation, synaptic loss and improved cognitive performance *in vivo* (Perez Ortiz and Swerdlow, 2019).

Nrf2 activators have not yet been tested in AD clinical trials. However, some of these compounds are being evaluated or have been approved for the treatment of other disorders and could be good candidates for AD due to their effective results in animal models. For example, sulforaphane has shown promising results against prostate cancer and dimethyl fumarate was approved for multiple sclerosis treatment (Bahn and Jo, 2019).

Natural antioxidants have also been subject of clinical research. This is the case of curcumin, whose oral supplementation to AD patients did not show any differences with placebo group (Jiang et al., 2016). Polyphenols have also been tested in clinical trials, being resveratrol and epigallocatechin gallate the most promising compounds, as they have reached phase III. Resveratrol did not display beneficial effects on AD patients, although it was found to cross the BBB (Turner et al., 2015; Zhu et al., 2018). On the other hand, no publications are available with epigallocatechin gallate results (Perez Ortiz and Swerdlow, 2019).

The inhibition of mPTP opening is also a potential mitochondrial target, as its blockage will improve mitochondrial function and reduce neuronal death. mPTP inhibitors have been developed, displaying positive effects on cellular models, but their clinical efficacy has not yet been determined (Elkamhawy et al., 2018)

- Anti-inflammatory strategies

In the past decades, epidemiological studies demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) consumption reduced AD risk. Particularly, a lower illness incidence was found in rheumatoid arthritis patients with long-term NSAIDs medication. These findings led to the use of rofecoxib, naproxen and indomethacin in AD clinical trials, but no clinical benefit was obtained. As these drugs are "classical" anti-inflammatory compounds, they are blocking both pro- and anti-inflammatory microglial response. This could have detrimental consequences to AD patients, in fact, an increasing risk of cardiac diseases was found in naproxen trial. Thus, the use of NSAIDs is not recommendable for the treatment of dementia (Ozben and Ozben, 2019).

The failure of NSAIDs trials produced a change of the anti-inflammatory approach for an immunomodulatory strategy, which is directed towards producing a shift in microglial phenotype without blocking their activity. GC-021109, a phagocytosis inducer, is currently in phase I clinical trials (Bachurin et al., 2017).

- Multitarget approach

The disappointing results obtained with clinical trials until now have led to a new perspective in AD therapeutics, a multitarget strategy. Most of the trials carried out were focused on one single target, and due to the complexity of the disease, it could be insufficient to face the pathology. Thus, a polypharmacological approach, which consists in simultaneously modulating multiple nodes of the disease networks, has emerged as a promising strategy for AD treatment (Rosini et al., 2016).

The multitarget strategy can be achieved both through a single drug able to affect to several targets or by combining various compounds that affect different pathways. There are many natural compounds that can be included in the first category, such as coumarin, curcumin and resveratrol (Gong et al., 2018). Although, as mentioned above, the main characteristic of these compounds is their antioxidant ability, they are known to modulate other AD hallmarks such as neuroinflammation, tau hyperphosphorylation or BACE1 activity (Tresserra-Rimbau et al., 2018).

Other approach is the design of multitarget-directed ligands, which has the aim to associate features that can interact with two or more targets. This can be afforded by combining different pharmacophore moieties from already identified bioactive molecules in one compound. The use of a single molecule can prevent the issues produced by simultaneously administering various drugs such as differences in bioavailability and pharmacokinetics. Initial efforts on multitarget development were focused on AchE inhibitors combined with anti-Aβ aggregation, BACE or GSK3β inhibition. Preclinical studies with these molecules have presented promising effects (Gong et al., 2018).

- Non-pharmacological interventions

In addition to therapeutic strategies directed towards stop the disease progression, preventive approaches have also been studied. Observational investigations have identified several modifiable risk factors for AD, such as hypertension, obesity, smoking, physical inactivity and low education levels. Their prevention through lifestyle interventions can result in beneficial effects on cognition, although the underlying mechanisms are not fully understood. Multidomain lifestyle trials, including dietary interventions, cognitive and exercise training, have been carried out and a beneficial effect on dementia occurrence was detected (Kivipelto et al., 2018).

Regular physical exercise has been reported to reduce the risk of AD development by 30-40%. In addition, physical exercise has been shown to enhance neurogenesis, synaptic plasticity, preserve brain volume and reduce ROS levels (Tari et al., 2019). Also, cognitive stimulating activities are related to a lower risk of suffering dementia, and low social contact is associated with an increased incidence of the disease (Kivipelto et al., 2018).

Regarding dietary interventions, several nutrients have been linked to a reduced cognitive impairment, such as vitamins C, D and E or polyunsaturated fatty acids. Among foods, regular fish, fruit and vegetable intake has been shown to produce a protective effect. Therefore, Mediterranean and Asiatic diets are associated with a lower risk of developing AD (Mendiola-Precoma et al., 2016).

We can conclude that the great efforts carried out on AD clinical research until now have displayed mostly disappointing results. This has been attributed to many reasons, such as the beginning of the illness decades before the appearance of first symptoms, the heterogeneous patient populations in clinical trials, and the intricate nature of the pathology, which cannot be faced with drugs focused on one single target (Anderson et al., 2017; Persson et al., 2014). Most of the compounds tested were directed towards Aβ and tau accumulation, and a change to a combination therapy or a multitarget strategy could to be a good starting point for AD treatment, as this kind of treatments have produced good results in other complex diseases such as cancer (Leon et al., 2013). In this sense, natural compounds possess a great potential as antioxidant and anti-inflammatory agents and have contributed to the search of new strategies for AD treatment (Habtemariam, 2019).

2. OBJECTIVE

Natural products, and particularly marine compounds, have a great therapeutic potential due to their great bioactivity and their effects at cellular level. Among those effects, it is noteworthy their ability to modulate the antioxidant defences and inflammatory pathways, making them promising candidates for drug development against diseases in which these events have an important role.

As a result of the great brain oxygen consumption, its limited defences against oxidative stress and the chronic activation of immune cells, neurodegenerative diseases such as AD are a clear example in which these natural compounds could be useful.

In this context, the objective of this doctoral thesis is the evaluation of 45 compounds with marine origin and 15 fruit-derived drinks to perform an initial determination of their potential as future therapeutic and preventive agents against AD:

- Evaluation of the neuroprotective ability of compounds and extracts in an *in vitro* model of oxidative stress
- Evaluation of the neuroprotective capacity of compounds and extracts in an *in vitro* model of neuroinflammation
- Evaluation of the effects of compounds on the main AD markers in cellular models
- Analysis of the anti-inflammatory effect of apple-derived functional drinks on a murine model of inflammation

3. PUBLICATIONS

This section contains the results obtained during this doctoral thesis, presented in ten research articles. The publications compiled the bioactivity data of marine-derived compounds and functional beverages.

Firstly, the bioactivities of cyanobacterial metabolites were analysed in human neuroblastoma cells. The results have been published in the following articles:

1. Structures and activities of Tiahuramides A-C, cyclic depsipeptides from a Tahitian collection of the marine cyanobacterium *Lyngbya majuscula*
2. Structure and biological evaluation of new cyclic and acyclic laxaphycin-A type peptides
3. Acyclic B-type laxaphycins induce AMPK-dependent autophagy in human neuroblastoma cells

Next, the neuroprotective potential of *Streptomyces* metabolites and sponge-derived compounds were determined in two cellular models of oxidative stress and neuroinflammation. In addition, the ability of bacteria-derived compounds to attenuate the main pathological markers of AD was evaluated *in vitro*. These data have been compiled in the following papers:

4. Streptocyclinones A and B ameliorate Alzheimer's disease pathological processes in vitro
5. Caniferolide A, a macrolide from *Streptomyces caniferus*, attenuates neuroinflammation, oxidative stress, amyloid-beta and tau pathology in vitro.
6. Evaluation of the antioxidant activity of the marine pyrroloiminoquinone makaluvamines
7. Evaluation of the protective effects of sarains on H_2O_2-induced mitochondrial dysfunction and oxidative stress in SH-SY5Y neuroblastoma cells
8. Simplified immunosuppressive and neuroprotective agents based on gracilin A
9. Gracilin A derivatives target early events in Alzheimer's disease: *in vitro* effects on neuroinflammation and oxidative stress

Finally, the protective ability of fruit-derived beverages was tested in the cellular models of oxidative stress and neuroinflammation, and four drinks were selected to determine their anti-inflammatory properties in a murine model of inflammation. These results are included in the following paper:

10. Neuroprotective effects of apple-derived drinks in a mice model of inflammation

The publications described in this section are presented below, with their corresponding abstracts.

1. Structures and activities of Tiahuramides A-C, cyclic depsipeptides from a Tahitian collection of the marine cyanobacterium *Lyngbya majuscula*

Abstract

The structures of three new cyclic depsipeptides, tiahuramides A (**1**), B (**2**), and C (**3**), from a French Polynesian collection of the marine cyanobacterium *Lyngbya majuscula* are described. The planar structures of these compounds were established by a combination of mass spectrometry and 1D and 2D NMR experiments. Absolute configurations of natural and nonproteinogenic amino acids were determined through a combination of acid hydrolysis, derivitization with Marfey's reagent, and HPLC. The absolute configuration of hydroxy acids was confirmed by Mosher's method. The antibacterial activities of tiahuramides against three marine bacteria were evaluated. Compound **3** was the most active compound of the series, with an MIC of 6.7 μM on one of the three tested bacteria. The three peptides inhibit the first cell division of sea urchin fertilized eggs with IC_{50} values in the range from 3.9 to 11 μM. Tiahuramide B (**2**), the most potent compound, causes cellular alteration characteristics of apoptotic cells, blebbing, DNA condensation, and fragmentation, already at the first egg cleavage. The cytotoxic activity of compounds **1**−**3** was tested in SH-SY5Y human neuroblastoma cells. Compounds **2** and **3** showed an IC_{50} of 14 and 6.0 μM, respectively, whereas compound **1** displayed no toxicity in this cell line at 100 μM. To determine the type of cell death induced by tiahuramide C (**3**), SH-SY5Y cells were co-stained with annexin V−FITC and propidium iodide and analyzed by flow cytometry. The double staining indicated that the cytotoxicity of compound **3** in this cell line is produced by necrosis.

https://pubs.acs.org/doi/10.1021/acs.jnatprod.7b00751

2. Structure and biological evaluation of new cyclic and acyclic laxaphycin-A type peptides

Abstract

Five new laxaphycins were isolated and fully characterised from the bloom forming cyanobacteria *Anabaena torulosa* sampled from Moorea, French Polynesia: three acyclic laxaphycin A-type peptides, acyclolaxaphycin A (**1**), [des-Gly^{11}]acyclolaxaphycin A (**2**) and [des-(Leu^{10}-Gly^{11})]acyclolaxaphycin A (**3**), as well as two cyclic ones, [L-Val^{8}]laxaphycin A (**4**) and [D-Val^{9}]laxaphycin A (**5**). The absolute configuration of the amino acids, established using advanced Marfey's analysis for compounds **2-5**, highlights a conserved stereochemistry at the Cα carbons of the peptide ring that is characteristic of this family. To the best of our knowledge, this is the first report of acyclic analogues within the laxaphycin A-type peptides. Whether these linear laxaphycins with the aliphatic β-amino acid on the *N*-terminal are biosynthetic precursors or compounds obtained after enzymatic hydrolysis of the macrocycle is discussed. Biological evaluation of the new compounds together with the already known laxaphycin A shows that [L-Val^{8}] laxaphycin A, [D-Val^{9}] laxaphycin A and [des-Gly^{11}] acyclolaxaphycin induce cellular toxicity whereas laxaphycin A and des-[(Leu^{10}-Gly^{11})] acyclolaxaphycin A do not affect the cellular viability. An analysis of cellular death shows that the active peptides do not induce apoptosis or necrosis but instead, involve the autophagy pathway.

https://www.sciencedirect.com/science/article/pii/S0968089618317139?via%3Dihub

3. Acyclic B-type laxaphycins induce AMPK-dependent autophagy in human neuroblastoma cells

Abstract

Introduction: Laxaphycins B are a family of non-ribosomal lipopeptides that have been isolated from several cyanobacteria species collected worldwide. Some of these compounds presented cytotoxic activities in previous works, but their mechanism of action is poorly understood. Moreover, the biological activities of acyclic laxaphycins B have not been tested so far. **Methods:** SH-SY5Y cells were treated with compounds (two cyclic and four acyclic laxaphycins) for 24 h and their effect on cell viability, mitochondrial function and reactive oxygen species release (ROS) was determined. Moreover, cells were stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry to determine the type of cell death induced by compounds, and caspase 3 activity was determined with a commercial kit. Finally, the expression of proteins involved in autophagy (mTOR, Beclin-1, LC3 and AMPK) was evaluated by western blot. **Results:** Cyclic laxaphycins B and B3 turned out to be cytotoxic (IC_{50}: 0.8 and 1.8 μM, respectively) through the induction of apoptosis, as evidenced by flow cytometry assays and caspase 3 activation. Otherwise, acyclic laxaphycins did not show cytotoxicity (up to 10 μM), but affected mitochondrial functioning and reduced ROS release. Moreover, these compounds increased the expression of autophagy-related enzymes. **Conclusion:** This work confirms the cytotoxicity of cyclic laxaphycins, providing evidences of an apoptotic process, and is the first description of the autophagy induction by their acyclic analogs. This process seems to be mediated by the ability of compounds to activate AMPK, a key regulator of the energetic state of cells.

4. Streptocyclinones A and B ameliorate Alzheimer's disease pathological processes *in vitro*

Abstract

Alzheimer's disease (AD) is a pathology characterized by the abnormal accumulation of amyloid-beta (Aβ) and hyperphosphorylated tau. Oxidative stress and neuroinflammation are also strongly related to this disease. The ability of two new glycosylated angucyclinones, streptocyclinones A and B (**1** and **2**), isolated from *Streptomyces* sp to improve AD hallmarks was evaluated. Compounds were able to protect SH-SY5Y neuroblastoma cells from H_2O_2-induced oxidative injury by activating the nuclear factor E2-related factor (Nrf2). Their capacity to modulate neuroinflammation was tested in lipopolysaccharide-activated BV2 microglial cells. Compounds reduced the release of pro-inflammatory factors, inhibited the activation of NFκB and mitogen activated kinases (MAPK), and induced the translocation of Nrf2 to the nucleus of microglial cells. A trans-well co-culture was established to determine the effect of microglia treated with streptocyclinones on the survival of SH-SY5Y cells. The cell viability of neuroblastoma cells increased when the compounds were added to BV2 cells. SH-SY5YTMHT441 cells were used to determine the effect of compounds on tau phosphorylation. Both compounds reduced tau hyperphosphorylation by targeting MAPK kinases. Moreover, streptocyclinone B (**2**) was able to inhibit the activity of β-secretase 1 and decrease the release of reactive oxygen species in BV2 cells stimulated with Aβ. With the same co-culture trans-well system, the treatment of Aβ-stimulated microglia with compound **2** augmented the viability of SH-SY5Y-TMHT441 cells. The results presented in this work provide evidences of the multitarget activities displayed by these new Streptomyces compounds, making them good candidates for further studies in the treatment of AD.

https://www.sciencedirect.com/science/article/pii/S0028390818306336?via%3Dihub

5. Caniferolide A, a macrolide from *Streptomyces caniferus*, attenuates neuroinflammation, oxidative stress, amyloid-beta and tau pathology *in vitro*

Abstract

The macrolide caniferolide A was isolated from extracts of a culture of the marine-derived actinomycete *Streptomyces caniferus*, and its ability to ameliorate Alzheimer's disease (AD) hallmarks was determined. The compound reduced neuroinflammatory markers in BV2 microglial cells activated with lipopolysaccharide (LPS), being able to block NFκB-p65 translocation to the nucleus and to activate the Nrf2 pathway. It also produced a decrease in pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), reactive oxygen species (ROS) and nitric oxide release and inhibited iNOS, JNK, and p38 activities. Moreover, the compound blocked BACE1 activity and attenuated Aβ-activation of microglia by drastically diminishing ROS levels. The phosphorylated state of the tau protein was evaluated in SH-SY5Y tau441 cells. Caniferolide A reduced Thr212 and Ser214 phosphorylation by targeting p38 and JNK MAPK kinases. On the other side, the antioxidant properties of the macrolide were determined in an oxidative stress model with SH-SY5Y cells treated with H_2O_2. The compound diminished ROS levels and increased cell viability and GSH content by activating the nuclear factor Nrf2. Finally, the neuroprotective ability of the compound was confirmed in two trans-well coculture systems with activated BV2 cells (both with LPS and Aβ) and wild type and transfected SH-SY5Y cells. The addition of caniferolide A to microglial cells produced a significant increase in the survival of neuroblastoma in both cases. These results indicate that the compound is able to target many pathological markers of AD, suggesting that caniferolide A could be an interesting drug lead for a polypharmacological approach to the illness.

https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.8b01090

6. Evaluation of the antioxidant activity of the marine pyrroloiminoquinone makaluvamines

Abstract

Makaluvamines are pyrroloiminoquinones isolated from *Zyzzya* sponges. Until now, they have been described as topoisomerase II inhibitors with cytotoxic effects in diverse tumor cell lines. In the present work, seven makaluvamines were tested in several antioxidant assays in primary cortical neurons and neuroblastoma cells. Among the alkaloids studied, makaluvamine J was the most active in all the assays. This compound was able to reduce the mitochondrial damage elicited by the well-known stressor H_2O_2. The antioxidant properties of makaluvamine J are related to an improvement of the endogenous antioxidant defenses of glutathione and catalase. SHSY5Y assays proved that this compound acts as a Nrf2 activator leading to an improvement of antioxidant defenses. A low concentration of 10 nM is able to reduce the reactive oxygen species release and maintain a correct mitochondrial function. Based on these results, non-substituted nitrogen in the pyrrole plus the presence of a *p*-hydroxystyryl without a double bond seems to be the most active structure with a complete antioxidant effect in neuronal cells.

https://www.mdpi.com/1660-3397/14/11/197

7. Evaluation of the protective effects of sarains on H_2O_2-induced mitochondrial dysfunction and oxidative stress in SH-SY5Y neuroblastoma cells

Abstract

Sarains are diamide alkaloids isolated from the Mediterranean sponge *Haliclona (Rhizoniera) sarai* that have previously shown antibacterial, insecticidal and anti-fouling activities. In this study, we examined for the first time the neuroprotective effects of sarains 1, 2 and A against oxidative stress in a human neuronal model. SH-SY5Y cells were coincubated with sarains at concentrations ranging from 0.01 to 10 µM, and the well-known oxidant hydrogen peroxide at 150 µM for 6 h and the protective effects of the compounds were evaluated. Among the sarains tested, sarain A was the most promising compound, improving mitochondrial function and decreasing reactive oxygen species levels in human neuroblastoma cells treated with the compound at 0.01, 0.1 and 1 µM. This compound was also able to increase the activity of the antioxidant enzymes superoxide dismutases by inducing the translocation of the nuclear factor E2-related factor 2 (Nrf2) to the nucleus at the lower concentrations tested (0.01 and 0.1 µM). Moreover, sarain A at 0.1 and 1 µM blocked the mitochondrial permeability transition pore (mPTP) opening through cyclophilin D inhibition. These results suggest that the protective effects produced by the treatment with sarain A are related with its ability to block the mPTP and to enhance the Nrf2 pathway, indicating that sarain A may be a candidate compound for further studies in neurodegenerative diseases.

https://link.springer.com/article/10.1007%2Fs12640-017-9748-3

8. Simplified immunosuppressive and neuroprotective agents based on gracilin A

Abstract

The architecture and bioactivity of natural products frequently serve as embarkation points for the exploration of biologically relevant chemical space. Total synthesis followed by derivative synthesis has historically enabled a deeper understanding of structure–activity relationships. However, synthetic strategies towards a natural product are not always guided by hypotheses regarding the structural features required for bioactivity. Here, we report an approach to natural product total synthesis that we term 'pharmacophore-directed retrosynthesis'. A hypothesized, pharmacophore of a natural product is selected as an early synthetic target and this dictates the retrosynthetic analysis. In an ideal application, sequential increases in the structural complexity of this minimal structure enable development of a structure–activity relationship profile throughout the course of the total synthesis effort. This approach enables the identification of simpler congeners retaining bioactivity at a much earlier stage of a synthetic effort, as demonstrated here for the spongiane diterpenoid, gracilin A, leading to simplified derivatives with potent neuroprotective and immunosuppressive activity.

https://www.nature.com/articles/s41557-019-0230-0

9. Gracilin A derivatives target early events in Alzheimer's disease: *in vitro* effects on neuroinflammation and oxidative stress

Abstract

The search for compounds capable of targeting early pathological changes of Alzheimer`s disease (AD), such as oxidative stress and neuroinflammation, is an important challenge. Gracilin A derivatives were recently synthesized, using a pharmacophore-directed retrosynthesis (PDR) strategy, and found to possess potent neuroprotective effects. In this work, the previously described derivatives **1-7** which had demonstrated mitochondrial-mediated, antioxidant effects were chosen for further study. The ability of compounds to modulate the expression of antioxidant genes (*CAT*, *GPx*, *SODs* and *Nrf2*) was determined in SH-SY5Y cells, and the simplified derivatives **2** and **3** were found to be the most effective. The anti-neuroinflammatory properties of derivatives were assessed in BV2 microglial cells activated with lipopolysaccharide (LPS). Several derivatives decreased the release of cytokines (Il-β, IL-6, GM-CSF and TNF-α) and other damaging molecules (ROS, NO) and also regulated the translocation of Nrf2 and NFκB, and reduced p38 activation. These protective effects were confirmed in a trans-well co-culture with BV2 and SH-SY5Y cells and several derivatives increased SH-SY5Y survival. This present work demonstrates the neuroprotective properties of gracilin A derivatives, making them promising candidate drugs for AD. Particularly, derivatives **2** and **3** showed the greatest potential as lead compounds for further development.

https://pubs.acs.org/doi/10.1021/acschemneuro.9b00329

10. Neuroprotective effects of apple-derived drinks in a mice model of inflammation

Abstract

Scope: Fruit-derived drinks consumption is considered beneficial due to the antioxidant and neuroprotective effects of polyphenols separately, but studies including their total constituents are scarce. In this work, we determined the antioxidant and anti-inflammatory neuroprotective effects of apple-derived beverages in a mouse model of LPS-induced inflammation.

Methods and results: Preliminary antioxidant and neuroinflammatory experiments were carried out with fifteen drink polyphenolic extracts in SH-SY5Y and BV2 cells, using H_2O_2 as pro-oxidant and LPS as pro-inflammatory stimulus, respectively. Extracts improved antioxidant systems functioning and presented neuroprotective mitochondrial-related effects. In microglia, extracts reduced reactive oxygen species and modulated cytokine release. To better mimic human consumption, four concentrated dealcoholized apple-derived drinks (three ciders and apple juice) were supplied to mice for seven days in substitution of drinking water. Mice treated with beverages presented reduced brain oxidative stress and inflammatory markers (lipid peroxidation, NO, TNF-α) after LPS injection. Interestingly, antioxidant enzymes genetic expression and glutathione levels were also greatly augmented after drink intake.

Conclusion: Our results confirm the antioxidant and anti-inflammatory-mediated neuroprotective properties of apple-derived drinks, suggesting that their consumption could be a good approach for the prevention of neurodegenerative disorders. To our knowledge, this is the first description of cider neuroprotective effects.

4. DISCUSSION

Cognitive decline and dementia prevalence are increasing worldwide, and they are projected to continue augmenting due to the aging of developed countries population. Therefore, neurodegenerative diseases constitute an emerging public health issue. Currently, about 50 million people are affected by dementia worldwide. This number is expected to double by 2030 and more than triple by 2050 (Alzheimer's-Association, 2016). Among dementias, AD is the most prevalent in elderly patients because it mainly affects people over 65 years and its incidence rate doubles each 5 years. The illness interferes with numerous aspects of human life including social, economic, physical, and psychological aspects, which worsens the economic burden. In fact, the World Health Organization has recently published a global action plan of public health response for 2017–2025, which includes a specific area for dementia as a public health priority focused on diagnosis, treatment, and care of these diseases (Cahill, 2019).

Although the understanding of several molecular pathways involved in AD pathology has increased, novel treatments capable of modifying the disease have not yet been achieved. This fact makes AD one important milestone for many researchers and pharmaceutical industries. There are several ongoing clinical trials for potential new drugs for AD, however, the results have been disappointing for now with a high failure rate of many lead drug candidates, specifically those aimed only at BACE1 (Moussa-Pacha et al., 2019). Due to the ineffectiveness of traditional AD therapeutic strategies, a shift towards new targets and, more particularly, to a multitarget strategy could have encouraging results (Cao et al., 2018).

In this sense, natural compounds, with their structural diversity, possess a great potential for AD treatment related to their antioxidant ant anti-inflammatory properties. In particular, the oceans harbour a great pool of undescribed molecules with unknown biological activities, so the research conducted through the elucidation of their mechanisms of action could be helpful for therapeutic approaches directed to pathologies that nowadays have no cure, such as AD (Liang et al., 2019).

In the present doctoral thesis, evidences have been provided about the neuroprotective effects of the marine-derived compounds tested, which were able to modify several pathological processes of the illness, highlighting their capacity to reduce oxidative stress and neuroinflammation. The metabolites preserved mitochondrial function, modulated microglial phenotypes, and even inhibited tau kinases and BACE1, providing a multifactorial response. Moreover, it was demonstrated that consumption of apple-derived drinks has a neuroprotective effect through the reduction of brain oxidative stress and inflammation.

A detailed discussion of each experimental work can be found within the peer-reviewed publications. Here is presented a general discussion that encompasses all the results obtained throughout this doctoral thesis.

Firstly, the results obtained with cyanobacterial compounds are presented. These photosynthetic organisms produce a great diversity of secondary metabolites, most of them non-ribosomal peptides with cytotoxic activities (Agrawal et al., 2017). Fourteen marine compounds obtained from this phylum were evaluated during the present dissertation: tiahuramides A, B and C, isolated from *Lyngbya majuscula*, and 11 laxaphycins derived from *Anabaena torulosa*.

With regard to the first compound family, tiahuramides B and C turned out to display cytotoxic effects, being the last one the most damaging molecule. Thus, it was disclosed by flow cytometry if this compound was inducing apoptotic or necrotic cell death, and it was found that a necrotic process was responsible of tiahuramide C toxicity.

The other family of cyanobacterial metabolites is divided in two sub-groups based on their structure: A-type and B-type laxaphycins. Here, five metabolites from the first sub-class and six from the second were analysed. It should be mentioned that two B-type compounds were obtained from an herbivorous gastropod, but the cyanobacteria can be considered their primary origin. The results obtained with this family of metabolites are summarized in Table 3.

Table 3. Summary of laxaphycins bioactivities in SH-SY5Y cells. The symbol ✓ indicates activity of the compound

Origin	Compound	Biological activities				
		Cytotoxicity	Cell death	↓ $\Delta\Psi_m$	↓ ROS	Autophagy
A. torulosa	Laxaphycin A			✓		
	[des-Gly^{11}] acyclolaxaphycin A	✓		✓	✓	✓
	[des-(Leu^{10}-Gly^{11})] acyclolaxaphycin A			✓		
	[L-Val^{8}] laxaphycin A	✓		✓		✓
	[D-Val^{9}] laxaphycin A	✓		✓	✓	✓
	Laxaphycin B	✓	Apoptosis	✓	✓	
	Laxaphycin B3	✓	Apoptosis	✓	✓	
	Acyclolaxaphycin B			✓	✓	✓
	Acyclolaxaphycin B3			✓	✓	✓
S. striatus	[des-(Ala^{4}-Hle^{5})] acyclolaxaphycin B			✓	✓	✓
	[des-(Ala^{4}-Hle^{5})] acyclolaxaphycin B3				✓	✓

Regarding A-type laxaphycins, [L-Val^{8}] laxaphycin A, [D-Val^{9}] laxaphycin A and [des-Gly^{11}] acyclolaxaphycin A showed cytotoxic effects, but flow cytometry analysis was not conclusive. In view of this results, it was determined if the decrease in cell viability observed was due to a different cellular event, autophagy. The activation of the autophagic flux occurs as a survival process under stress conditions, but when it is excessive it can result in cellular death due to an extreme self-digestion (Doherty and Baehrecke, 2018). The expression analysis of autophagic flux key proteins revealed that those laxaphycins were activating this pathway, as evidenced by the increase in LC3 II levels, a marker of autophagosome maturation, and the decrease in mTOR phosphorylation, a negative autophagy regulator (Ravanan et al., 2017). Moreover, these laxaphycins altered p62 expression, a protein with a critical role in selective autophagy (Liu et al., 2017). Due to the effect of these compounds both in p62 and $\Delta\Psi_m$, the results point to a mitophagic process, through which damaged mitochondria are removed (Liu et al., 2017). With respect to B-type laxaphycins, these metabolites showed a clear structure-activity relationship. Cyclic B and B3 peptides were cytotoxic through apoptosis, as confirmed by flow cytometry analysis and the increase in caspase-3 activity. Otherwise, acyclic compounds activated the autophagic flux, evidenced by the augmented expression of LC3 II and beclin 1. In this case, the metabolites increased AMPK phosphorylation, whose activity provides energy and intermediaries for the synthesis of new molecules (Tamargo-Gomez and Marino, 2018). Interestingly, biotransformation does not seem to affect the bioactivity of acyclic laxaphycins, since the compounds isolated from *Stylocheilus striatus* retained their ability to stimulate the autophagic flux, which could be conferring the animal some ecological advantage.

Because of the cytotoxicity displayed by tiahuramides, A-type laxaphycins and cyclic laxaphycins B, these compounds were discarded for neuroprotection assays. However, acyclic B-type laxaphycins could be useful for AD treatment, since autophagy is dysregulated in the illness and its non-excessive activation represents a major pathway for clearance of aggregated proteins and dysfunctional organelles. Particularly, AMPK activation increases cellular energy and prevents energetic failure, being of great importance in neurodegenerative disorders (Morgunova and Klebanov, 2019).

Following with prokaryotic-derived compounds, the neuroprotective potential of three metabolites from *Streptomyces* strains was evaluated. These molecules belong to two different families, streptocyclinones A and B were new compounds with unknown bioactivities, whereas caniferolide A is a macrolide that had displayed anti-fungal properties (Perez-Victoria et al., 2019).

Since AD is highly related to ROS accumulation and mitochondrial alterations, we first evaluated the antioxidant ability of these molecules in an *in vitro* model of oxidative stress. The oxidative imbalance observed in neurodegeneration was mimicked by adding the oxidant H_2O_2 to neuroblastoma cells. Several parameters were measured to determine the protective effect of the secondary metabolites, such as ROS levels, mitochondrial function or the state of antioxidant systems. Actinobacteria-derived compounds were able to protect cells from oxidative damage, augmenting GSH levels and reducing ROS. Their antioxidant effects were mediated by the translocation of Nrf2 to the nucleus.

In view of these promising results, the metabolites were tested in a cellular model of neuroinflammation, which was established with the murine microglial cell line BV2. In order to trigger the inflammatory response in these cells, a component of the outer membrane of Gram-negative bacteria was used, lipopolysaccharide (LPS). The addition of LPS activates the inflammatory cascade, producing the release of toxic molecules such as NO and cytokines, and the stimulation of cellular pathways involved in neuroinflammation such as NFkB or MAPKs (Alam et al., 2019; Jia et al., 2019). The three *Streptomyces* metabolites reduced the release of pro-inflammatory mediators and the activation of enzymes involved in the inflammatory response. Importantly, both streptocyclinones and caniferolide A augmented the anti-inflammatory cytokine IL-10 and translocated Nrf2 to the nucleus, as it happened in neuronal cells. Together, these results suggest an immunomodulatory ability of the metabolites that was finally confirmed with a co-culture between both cell lines. In this system, microglial cells were treated with *Streptomyces* compounds and a meaningful protective effect was observed in neuroblastoma cells, whose cell survival was improved in the presence of metabolites. *Streptomyces*-derived compounds results, both in oxidative stress and neuroinflammation cellular models, are presented in Table 4.

Table 4. Summary of the results obtained with *Streptomyces* compounds in oxidative stress and neuroinflammation cellular models. The symbol ✓ indicates positive activity of the compound

Origin	Compound	Oxidative stress model					Neuroinflammation model						
		↑ Cell viability	↑ $\Delta\Psi_m$	↓ ROS	GSH	Nrf2	Pro-inflammatory mediators	IL-10	NFkB	Nrf2	iNOS	MAPKs	Co-culture
Streptomyces sp.	Streptocyclinone A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Streptocyclinone B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Streptomyces caniferus	Caniferolide A	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Due to the positive effects of actinomycetes compounds, their capacity of modulating AD specific hallmarks was analysed (Table 5). For tau hyperphosphorylation analysis, a transfected neuroblastoma cell line was used, SH-SY5Y-TMHT441 cells. These cells have two human mutations present in tauopathies that result in tau hyperphosphorylation in AD-related residues. Tau phosphorylated status was analysed with specific antibodies, obtaining positive results with all *Streptomyces*-derived metabolites, which reduced tau hyperphosphorylation at some of the residues examined. This decrease was mediated by their capacity to modulate MAPKs activation, a property previously observed in microglial cells. In addition, BACE1 enzymatic activity was monitored in a non-cellular assay with compounds. Streptocyclinone B and the macrolide produced a dose-dependent inhibition of the enzyme, so these metabolites were selected to determine whether they could have anti-neuroinflammatory properties in microglial cells activated with Aβ fibrils. Both compounds decreased microglial ROS release, so a new co-culture was performed. In this case, Aβ-activated microglia was seeded in inserts placed above transfected neuroblastoma cells, and once again, streptocyclinone B and caniferolide A presented protective effects.

Table 5. Results of *Streptomyces*-derived metabolites in AD hallmarks

Origin	Compound	Tau					Aβ		
		Phosphorylated residues	p38	ERK	JNK	GSK3B	BACE1	Microglial ROS	Co-culture
Streptomyces sp.	Streptocyclinone A	✓		✓	✓			ND	ND
	Streptocyclinone B	✓	✓		✓		✓	✓	✓
Streptomyces caniferus	Caniferolide A	✓	✓		✓	ND	✓	✓	✓

With respect to natural sponge compounds, one metabolite from a specimen of *Zyzzya* sp. collected in Fiji Islands, makaluvamine J, and three from the Mediterranean sponge *Haliclona* *sarai*, sarains 1, 2 and A, were tested in the *in vitro* model of oxidative stress described before. The first metabolite was the most effective of seven compounds isolated from the same family in an oxidative stress model with murine cortical neurons, so it was selected to test its neuroprotective activity in a human model. As expected, makaluvamine J also protected human cells from H_2O_2 damage, producing a recovery of $\Delta\Psi_m$ and improving the functioning of antioxidant systems. Nrf2 expression analysis confirmed that this effect was due to its translocation to the nucleus. This makaluvamine was the most active compound compared to its congeners, which have a closely related chemical structure. In particular, makaluvamine P structure, with a poor activity, is almost identical to makaluvamine J. The presence of a pyrrole with a non-substituted nitrogen is the only difference, so it seems to be essential for the antioxidant activity of the compound. Regarding sarains, their chemical characteristics are also related to their therapeutic potential, since sarain A presented very promising results against oxidative stress, whereas sarains 1 and 2 did not show activity in this model. The presence of a masked aldehyde group that can react with proteins in sarain A has been proposed to be responsible for its greater activity (Defant et al., 2011). The protective properties of sarain A, which enhanced mitochondrial function and SODs activity, were ‘mediated by Nrf2, but this metabolite also blocked mPTP opening. The compound was found to inhibit CypD activity, the main enzyme involved in the pore triggering, preventing its opening and producing the consequent amelioration of mitochondrial function.

The experiments with marine-derived compounds were finished with the evaluation of 24 synthetic derivatives of gracilin A, a sponge secondary metabolite. The compound belongs to a family with interesting neuroprotective and immunosuppressive properties (Leiros et al., 2015; Leirós et al., 2014; Sanchez et al., 2016a; Sanchez et al., 2016b), so it was selected to perform a PDR. This synthetic approach, which selects a pharmacophore as synthetic target and sequentially increases the compounds structural complexity, was carried out to identify the simplest compounds that conserved the biological activities observed in the natural molecule. During this doctoral thesis, the neuroprotective properties of derivatives were determined, both in oxidative stress and neuroinflammation *in vitro* models. With regard to their antioxidant ability, compounds 21a, 27a and 27b, with simplified structures, were the most effective derivatives, along with the more complex molecules 29a, 21b, 22 and 23c, the latter being the closest to gracilin A structure. They improved mitochondrial function, evidenced by $\Delta\Psi_m$ recovery, and most of them reduced ROS levels and recuperated GSH content. In view of these results, the seven derivatives were selected to test their ability to block mPTP opening, a property of their natural scaffold. Five derivatives (27a, 27b, 29a, 21b and 23c) prevented the initiation of the pore through CypD inhibition, being remarkable the selectivity of 27a, 27b and 29a for this mitochondrial isoform over CypA. Finally, the capacity of gracilin A derivatives to modulate the expression of antioxidant genes was determined. All the compounds were found to upregulate some of the antioxidant genes analysed, being derivatives 27a and 27b the most effective. These molecules, with simplified structures compared to gracilin A, were particularly efficient in all the assays and, together 29a, showed a distinctive feature of the natural compound, they were selective inhibitors of CypD activity over CypA. Compounds that inhibit CypD usually affect to the cytosolic isoform CypA, and the identification of CypD selective inhibitors is outstanding because they will have neuroprotective effects without altering immunosuppressive pathways (Valasani et al., 2014). In fact, the most selective compound, 27b, was tested in preliminary immunosuppressive assays and no activity was detected. The results of selected gracilin A synthetic derivatives in oxidative stress assays are summarized in Table 6.

Table 6. Activity of selected gracilin A synthetic derivatives in the oxidative stress model. The symbol ✓ indicates positive activity of the compound

Origin	Compound	Oxidative stress model								
		↑ Cell viability	↑ $\Delta\Psi_m$	↓ ROS	mPTP blockade	Genetic expression				
						CAT	SOD1	SOD2	GPx1	Nrf2
Gracilin A PDR	21a	✓	✓	✓		✓		✓		
	27a	✓	✓	✓	✓	✓	✓		✓	✓
	27b	✓	✓	✓	✓	✓	✓	✓	✓	✓
	29a	✓	✓	✓	✓			✓	✓	
	21b	✓	✓					✓	✓	✓
	22	✓	✓	✓	✓			✓	✓	✓
	23c	✓	✓	✓			✓	✓	✓	✓

Selected gracilin A derivatives also displayed immunomodulatory properties in the assays with microglial cells. Derivatives modulated Nrf2 and NFkB, augmenting the neuroprotective transcription factor and inhibiting the pro-inflammatory one. The immunomodulatory effect was further confirmed when pro-inflammatory mediators were measured. Compounds reduced ROS, NO and pro-inflammatory cytokines release, with a great effect on IL-1β, considered the initiating cytokine of the inflammatory cascade (Rubio-Perez and Morillas-Ruiz, 2012). Finally, the microglia-neuroblastoma co-culture confirmed the neuroprotective ability of gracilin A derivatives, since the seven compounds protected neuronal cells from microglial damage.

Among the selected synthetic compounds, 27a and 27b presented the best results, followed by 29a. As mentioned before, these derivatives were selective inhibitors of CypD, so the effects obtained in microglial cells suggest an involvement of the PPIase in their activation. The role of CypD beyond the mPTP opening remains unknown (Porter and Beutner, 2018), but CypD-deficient macrophages have presented a decreased inflammatory response, evidencing the role of CypD and mPTP in inflammation (Priber et al., 2015). Thus, it is expected that CypD inhibition in microglial cells has similar implications, since they are the macrophages of CNS. Our results with derivatives open an interesting field to explore the influence of CypD in the activation of microglia.

To finalize the marine-derived compounds section, it is important to highlight that the results obtained in these assays with some compounds, both natural and synthetic, were better at lower concentrations, the metabolites did not show a dose-dependent effects. This behaviour has been reported in natural compounds such as curcumin or resveratrol, which present antioxidant effects at low doses and induce oxidative stress and cell death at high concentrations (Moghaddam et al., 2019; Posadino et al., 2015). With regard to inflammation, some compounds produce a similar response, inducing a polarization towards the anti-inflammatory phenotype at low doses and activating the pro-inflammatory state at higher concentrations (Calabrese et al., 2018). In this way, the metabolites evaluated could be interacting with other cellular pathways at high concentrations, or with a receptor that could be suffering a threshold effect, that is, it would present higher affinity at low concentrations and would be desensitized at higher doses (Williams and Spencer, 2012).

Finally, within the framework of a national research project co-financed by Ministerio de Economía, Industria y Competitividad and FEDER funds, the antioxidant and anti-neuroinflammatory capacity of 15 fruit-derived drinks was determined. Most of the studies found in literature evaluated fruit compounds separately (Braidy et al., 2016; Lee et al., 2013), but these molecules appear combined in nature. Thus, the whole polyphenolic content of fruit-derived beverages was extracted to assess their neuroprotective effects against oxidative stress and neuroinflammation. Importantly, beverage foam and gas were removed, and ethanol was evaporated before obtaining polyphenolic extracts. Four carbonated juices (cocoa, aloe, acai and apple), three wine mixes (sangria, 'tinto de verano' and 'tinto de verano' with lemon), two musts (red and white) and six ciders (pear, sweet, dry, strawberry, blackberry and natural) from a local industry were included in the study. Polyphenolic extracts presented great *in vitro* antioxidant properties, decreasing ROS levels, recovering $\Delta\Psi_m$ and GSH content, and improving SODs activity. The results of carbonated juices and some cider extracts on ROS levels were noteworthy, since they reduced the toxic molecules by 50 %. In addition, cider extracts presented the best results on mitochondrial function. Regarding neuroinflammation, the effects were smaller, but some drink extracts turned out to have promising properties, such as carbonated juices, red wine mixes, and pear and sweet ciders, which generally decreased ROS and TNF-α, and augmented IL-10 levels.

Based on cellular data, four drinks were chosen to perform *in vivo* experiments in a mice model of inflammation: apple carbonated juice, and pear, sweet and dry ciders. Despite the good results obtained with red wine mixes and musts, we chose apple drinks because of the extensive research carried out with resveratrol, a major component of grape beverages (Braidy et al., 2016). Concentrated dealcoholized drinks were provided to mice for one week in substitution of drinking water, and the last day of treatment LPS was administered to the animals by intraperitoneal injection to induce a systemic inflammatory response. LPS injection, together with the increase of inflammatory markers, also generates a decrease in GSH and an augmentation of lipid peroxidation in the brain (Al-Amin et al., 2018). Thus, this model is a good tool for assessing brain oxidative stress and neuroinflammation *in vivo*. It was found that drinks consumption increased the expression of antioxidant genes such as SOD1 and CAT in mice brains, but surprisingly, when their enzymatic activity was analysed, a decrease was detected. These results suggest that the drinks are able to directly neutralize ROS, resulting in a deactivation of antioxidant systems, so the beverages would have a double antioxidant activity, both directly and indirectly, which has been reported for many polyphenols (Renaud and Martinoli, 2019). A reduction in lipid peroxidation was also found in brains of drink-treated mice, along with a decrease in the pro-inflammatory marker NO. Regarding cytokines release, brain IL-10 was augmented only by sweet cider, but TNF-α levels were decreased by the drinks, especially in blood. Different effectiveness was detected with the beverages analysed, being apple juice the best drink, followed by pear and sweet ciders (Table 7). Otherwise, dry cider did not display protective results, although it was able to upregulate antioxidant gene expression. In view of these results, juice addition seems to be a good strategy to improve cider neuroprotective properties, and apple-derived drinks consumption could be a good preventive approach against neurodegeneration, since oxidative stress and neuroinflammation are known to occur in early AD stages (Streit et al., 2018; Wang et al., 2014).

Table 7. Summary of *in vivo* results with selected apple-derived drinks. The symbol ✓ indicates positive activity of the compound.

Drink	***In vivo* inflammation model**									
	Antioxidant gene expression	↓ SOD's activity	↓ CAT activity	GSH	MDA	NO	Brain		Serum	
							IL-10	TNF-α	IL-10	TNF-α
Apple juice	✓	✓	✓	✓	✓	✓		✓		✓
Pear cider	✓		✓	✓	✓	✓		✓		✓
Sweet cider	✓		✓		✓	✓	✓			✓
Dry cider	✓									✓

5. CONCLUSIONS

1. Tiahuramides B and C, obtained from the cyanobacteria *Lyngbya majuscula*, are cytotoxic in human neuroblastoma cells. The most toxic metabolite, tiahuramide C, produces a necrotic cell death.

2. Three A-type laxaphycins, [des-Gly^{11}] acyclolaxaphycin A, [L-Val^{8}] laxaphycin A and [D-Val^{9}] laxaphycin A, isolated from *Anabaena torulosa*, produce an excessive activation of the autophagic flux, leading to a reduction in cell viability.

3. Cyclic B-type laxaphycins induce apoptosis, whereas acyclic members of this sub-family produce AMPK-dependent autophagy.

4. The sponge-derived compound makaluvamine J protects human neuroblastoma cells from oxidative stress, reducing ROS levels and increasing antioxidant systems activity through the activation of Nrf2.

5. Sarain A, isolated from a Mediterranean sponge, improves mitochondrial function and decreases ROS levels due to its ability to block mPTP opening and to activate the transcription factor Nrf2.

6. The *Streptomyces*-derived compounds streptocyclinones and caniferolide A protect human neuroblastoma cells from oxidative stress, producing a reduction in ROS and an enhancement of GSH content via Nrf2-ARE pathway. Streptocyclinones also ameliorate mitochondrial function.

7. Microglial phenotypic state is modulated by *Streptomyces* compounds in a murine microglial cell line, provoking a reduction in the release of pro-inflammatory molecules. This effect is mediated by the regulation of the enzymes involved in the inflammatory response MAPKs, NFkB and Nrf2, and leads to the protection of neuroblastoma cells in a co-culture system.

8. Streptocyclinones and caniferolide A reduce tau phosphorylation through the modulation of MAPKs in the cellular model SH-SY5Y-TMHT441. Moreover, streptocyclinone B and caniferolide A inhibit BACE1 activity and decrease ROS release by Aβ-activated microglia, improving the survival of tau-transfected neuronal cells in a co-culture.

9. Seven gracilin A derivatives, 21a, 27a, 27b, 29a, 21b, 22 and 23c, show neuroprotective effects against oxidative stress by their ability to upregulate the expression of antioxidant genes. Compounds 27a, 27b, 29a and 22 block mPTP opening by inhibiting CypD enzymatic activity.

10. Derivatives 27a, 27b and 29a present selective CypD activity inhibition over CypA.

11. Gracilin A-derived compounds reduce NFkB translocation and increase Nrf2 activation in microglial cells, decreasing the release of pro-inflammatory mediators and protecting human neuroblastoma cells from microglial damage in a trans-well co-culture.

12. Apple-derived drinks show neuroprotective effects in a murine model of inflammation through the reduction of oxidative stress and pro-inflammatory markers.

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