

SIMPLER: Fungi-to-human drug screening platforms towards the treatment of neurodegenerative disorders

Francisca de Sousa Coelho

Integrated Master in Bioengineering – Molecular Biotechnology

Supervisor: Sofia Guimarães, PhD (i3S/ INEB)

Co-supervisor: Ana Paula Pêgo, PhD (i3S/ INEB/ ICBAS)

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“Everything is theoretically impossible, until it is done”

-Robert A. Heinlein

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Abstract

Neurodegenerative disorders represent a group of conditions such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis that are responsible for substantial medical and public health burdens worldwide. Millions are diagnosed with a neurodegenerative condition every year and with the aging of the population the numbers are expected to continue to rise. Currently, there is an urgent need for treatments since there is no cure and the treatments available rely solely on easing some physical and mental symptoms.

Studies have been demonstrating that loss of microtubules mass, dysfunction microtubules dynamic and microtubules instability are associated with neurodegenerative disorders. This discovery has propelled the development of microtubule-stabilizing drugs that may counteract neurodegeneration by restoring the correct functioning of microtubules.

Drug development is long, costly, and with a low success rate. Consequently, recently, drug repurposing has emerged as a strategy to identify new targets for approved drugs outside of their original application to accelerate new treatments to the bedside. Drug repurposing has the advantage of the existence of bioactivity and safety information in humans, accelerating drug development. Further, the quest for novel microtubule-stability agents would benefit from the existence of a simple low-cost model, amenable, that surpasses the complexity of models that emulate the central nervous system.

Ustilago maydis, a dimorphic filamentous fungus shows phenotypically similarities with neurons and has conserved microtubules mechanisms. Our team has previously proposed *U. maydis* as a suitable whole-organism model for high-throughput screening towards microtubules modulation.

Previously in the group, it was performed the high-throughput screening of Prestwick chemical library in *U. maydis* model, and 22 positive hits were obtained. Here, an *in silico* analysis of these positive hits is performed. From those, five (Aprepitant, Atracurium besylate, Danazol, Disulfiram, and Pranlukast) were chosen to pursue with validation in the mammalian system. Several tools were developed to validate the compounds: i) Differentiated ND7/23 cell line (immortalized dorsal root ganglion neuron) for image analysis through morphology and collar occupancy method; ii) mouse primary cortical neurons using cell morphology analysis and average skeleton length, and iii) mouse primary cortical neurons seeded in a microfluidic device to evaluate cell morphology. Aprepitant, Atracurium besylate, Disulfiram and Pranlukast, displayed in all studied models, some degree of microtubule-stability capacity. Among these, Atracurium besylate showed a higher microtubule-stability capacity than Taxol, a known microtubule-stabilizer.

Therapies targeting neurological disorders are hampered by the selective nature of the blood-brain barrier. Here, we present the initial steps for the implementation of a BBB-on-a-chip device for the evaluation of drugs permeability into central nervous system. The BBB-on-a-chip construct was made of two poly(dimethylsiloxane) microfluidic intercalated by a porous poly(ethylene terephthalate) membrane – representing the basal membrane. Through 3D printing technology, templates for the two microfluidic were printed using a custom-made

method. This technology allowed the optimization of the device throughout the work necessities.

For tailoring the poly(dimethylsiloxane) and poly(ethylene terephthalate) surfaces for improvement of cell adhesion, 3 approaches were studied: extracellular matrix protein adsorption, plasma treatment, and polydopamine coating. Although none of the coatings showed the expected efficacy in cell adhesion, the best approach for modifying the surfaces would be combining plasma treatment followed by poly-D-lysine in the poly(ethylene terephthalate) membrane and polydopamine treatment followed by the collagen coating in the endothelial microfluidic. When completely established it will contribute to further validate the hits arising from the high-throughput screening.

In conclusion, we report evidence that *U. maydis* can be a proper high-throughput screening model for evaluation of microtubule-stability agents, accelerating the drug development process. Moreover, with the mammalian tools developed in this project, it was possible to validate the proposed fungus-based drug screening platform. Moreover, Atracurium besylate was found to be a potential candidate for microtubule modulation towards the treatment of neurodegenerative disorders.

Keywords: neurodegeneration; *Ustilago maydis*; drug repurposing, high-throughput screening platforms; BBB-on-a-chip.

Sumário

As doenças neurodegenerativas são constituídas por um grupo de variado de patologias como a doença de Alzheimer, Parkinson e esclerose lateral amiotrófica, responsáveis por substanciais encargos médicos e de saúde pública em todos os países do mundo. Todos os anos milhões de pessoas são diagnosticadas com uma doença neurodegenerativa e com o envelhecimento da população é expectável que os números continuem a aumentar. Atualmente é urgente a necessidade de tratamentos para estas patologias uma vez que não existe cura e as opções de terapia baseiam-se apenas no alívio de alguns sintomas físicos e mentais.

Vários estudos têm demonstrado que associado a alterações neurodegenerativas está a perda de massa dos microtúbulos e a alteração da sua dinâmica e a instabilidade. Esta descoberta impulsionou o desenvolvimento de drogas estabilizadoras de microtúbulos que ao restaurar o correto funcionamento dos microtúbulos podem contrariar a neurodegeneração.

O desenvolvimento de medicamentos é um processo longo, caro e com baixa taxa de sucesso. Para tentar contrariar algumas destas adversidades, recentemente, o redireccionamento de medicamentos surgiu como uma estratégia para identificar novos alvos terapêuticos para medicamentos já aprovados por agências reguladoras. A grande vantagem do redireccionamento de fármacos é a existência de informações de segurança em humanos, o que permite acelerar o processo de desenvolvimento, encurtando etapas. Para além do aproveitamento do método de redireccionamento de fármacos, a procura por novos agentes estabilizadores de microtúbulos beneficiaria da existência de um modelo simples, de baixo custo e que superasse a complexidade dos modelos que representam sistema nervoso central.

O *Ustilago maydis* é um fungo filamentosso dimórfico que para além de apresentar semelhanças fenotípicas com neurónios de mamíferos também tem a mais valia de os mecanismos de microtúbulos serem conservados entre este fungo e humanos. *U. maydis* foi anteriormente proposto pela nossa equipa como um modelo de organismo inteiro adequado para a avaliação de drogas capazes de modular os microtúbulos.

Assim, previamente no grupo foi realizado um estudo da biblioteca química Prestwick no modelo *U. maydis* e desse trabalho foram obtidos vinte e dois candidatos positivos (com capacidade para estabilizar os microtúbulos). Neste projeto foi então realizada uma análise *in silico* dos candidatos positivos. Dos vinte e dois, cinco candidatos positivos (Aprepitant, Atracurium besylate, Danazol, Disulfiram e Pranlukast) foram escolhidos para prosseguir para uma validação em modelos de mamíferos. Várias ferramentas foram desenvolvidas para validar estes compostos: i) Linha celular ND7/23 diferenciada para análise de imagem através de morfologia e do método de “collar occupancy”; ii) neurónios corticais primários de ratinho recorrendo a análise de morfologia celular e comprimento médio do esqueleto; e iii) neurónios corticais de ratinho em cultura num dispositivo de microfluídica para avaliar a morfologia celular. Aprepitant, Atracurium besylate, Disulfiram e Pranlukast apresentaram em todos os modelos estudados alguma capacidade de estabilidade dos microtúbulos. Destes, o Atracurium besylate foi o composto que apresentou maior capacidade para estabilizar os microtúbulos, tendo ultrapassado o Taxol, uma droga já estabelecida como estabilizadora dos microtúbulos.

As terapias direcionadas às doenças neurodegenerativas são dificultadas pela natureza seletiva da barreira hematoencefálica. Por isso, neste projeto, foram apresentadas as etapas iniciais para a implementação de um dispositivo BBB-on-a-chip para avaliação da permeabilidade de drogas no sistema nervoso central. A construção deste sistema foi feita recorrendo a duas microfluídicas de poli(dimetilsiloxano) intercaladas por uma membrana porosa de poli(tereftalato de etileno) – que representa a membrana basal. Através da tecnologia de impressão 3D, os moldes usados para a produção das microfluídicas foram impressos de forma personalizada. Esta tecnologia permitiu a otimização do dispositivo ao longo do desenvolvimento do projeto e de acordo com as necessidades deste.

Para modificar as superfícies de poli(dimetilsiloxano) e poli(tereftalato de etileno) de maneira a melhorar a adesão celular, três métodos foram estudados: adsorção de proteínas da matriz extracelular, tratamento com plasma e revestimento de polidopamina. Embora nenhuma das modificações tenha mostrado eficácia na adesão celular, a melhor abordagem para modificar as superfícies seria combinar o tratamento de plasma seguido por adsorção de poli-D-lisina na membrana de poli(tereftalato de etileno) e tratamento de polidopamina seguido pela adsorção de colagénio na microfluídica endotelial. Quando este modelo BBB-on-a-chip tiver totalmente estabelecido poderá contribuir para validar ainda mais os candidatos positivos da análise de drogas no modelo *U. maydis*.

Concluindo, neste projeto foram apresentadas evidências de que *U. maydis* pode ser um modelo para avaliação de possíveis candidatos a agentes estabilizadores dos microtúbulos, permitindo assim acelerar o processo de desenvolvimento de drogas. Além disso, com as ferramentas de modelos de mamíferos estabelecidas aqui foi possível validar o fungo *U. maydis*. Para além disso, descobriu-se que o Atracurium besylate é um forte candidato a estabilizador dos microtúbulos e, portanto, como uma potencial droga para o tratamento de doenças neurodegenerativas.

Palavras-chave: neurodegeneração; *Ustilago maydis*; redireccionamento de medicamentos; BBB-on-a-chip

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Abbreviations

AJ	A dherent j unction
AD	A lzheimer's d isease
ALS	A myotrophic lateral s clerosis
Aβ	A myloid-beta
BBB	B lood- b rain b arrier
BSA	B ovine serum a lbumin
CNS	C entral n ervous s ystem
CM	C omplete m edium
CAD	C omputer- a ided d esign
DIV	D ays <i>in vitro</i>
DA	D opamine
DRG	D orsal R oot G anglia
DMEM	D ulbecco's m odified e agle m edium
EC	E ndothelial c ell
EpoD	E pothilone D
ECM	E xtracellular m atrix
FBS	F oetal b ovine serum
GDP	G uanosine-5'- d iphosphate
GTP	G uanosine-5'- t riphosphate
HBSS	H ank's B alanced S alt S olution
HSANs	H ereditary sensory and a utonomic n euro p athies
HSP	H ereditary s pastic p araplegia
HTS	H igh-throughput screening
HD	H untington d isease
JAM	J unction a dhesion m olecules
MPTP	1- m ethyl-4- p henyl-1,2,3,6- t etrahydropyridine

MT	Microtubules
MAP	Microtubule-associated protein
MTOC	Microtubule-organizing center
MT-PFA	Microtubule-paraformaldehyde
MGCs	Mixed glial cells
NVU	Neurovascular unit
NGF	Nerve growth factor
NM	Nitrate minimal
OPCs	Oligodendrocyte progenitor cells
P-gp	P-glycoprotein
PD	Parkinson's disease
P/S	Penicillin-Streptomycin
PNS	Peripheral nervous system
PBS	Phosphate buffered saline
PDL	Poly(-D-lysine)
PDMS	Poly(dimethylsiloxane)
PDA	Poly(dopamine)
PET	Poly(ethylene terephthalate)
PTM	Post-translational modification
ROI	Region of interest
RNA	Ribonucleic acid
SLD	Second-level domain
SD	Standard-deviation
TJ	Tight junction
TIP	Tracking protein
TEER	Trans-endothelial electrical resistance
Tg	Transgenic

RPM	R otations p er m inute
SEM	S canning e lectron m icroscopy
WD	W allerian d egeneration

Motivation

Neurodegenerative disorders are responsible for substantial medical and public health burdens on populations worldwide. These pathologies affect millions of people, and being ageing a risk factor, the number of cases is expected to rise with average life expectancy increasing [1].

Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are three of the major neurodegenerative diseases. The treatments available enables to relieve some of the physical and mental symptoms, but currently, there is no cure or even therapy to slow disease progression [2]. Thus, the need for a treatment is urgent, with drug repurposing possibly being a faster and viable solution towards this goal.

Genetic, *in vitro*, and *in vivo* studies have identified the loss of microtubules (MTs) mass and function, alteration in MT dynamics or stability, and disturbance of the MT-mediated transport in various unrelated neurodegenerative diseases [3-5]. MTs are one of the major cytoskeletal components essential for many fundamental cellular and development processes. Since neurons are very susceptible to MTs regulation, it is not surprising that MT dysfunctions may have a contribution to the onset of neurodegenerative disorders [6].

These discoveries propel the development of compounds that counteract neurodegeneration by restoring the correct functioning of MTs, namely MT-stabilizing drugs. Until now, *in vivo* studies suggested that MT-stabilizing agents might provide a viable therapeutic strategy [7]. Paclitaxel also known as "Taxol", an anti-cancer drug, was the first reported compound to stabilize MTs in neurodegenerative like-pathology mouse models [5, 6]. Despite the beneficial effects, paclitaxel was shown to be an unfit therapeutical candidate for neurodegenerative diseases due to its limited brain bioavailability.

Therefore, since neurodegeneration diseases affect specific areas of the brain, brain-penetrant MT-stabilizing compounds are required. Recently, studies have focused on the therapeutic effect of epothilone D (EpoD), a promising MT-stabilizing agent with blood-brain barrier (BBB) permeability [7]. Regardless, translational into clinical was not successful, with clinical trials being interrupted after phase I [6].

Thereby, there is an emergent need to identify additional MT-stabilizing drugs with BBB permeability and prolonged brain retention. The quest for novel microtubule-stability agents would benefit from the existence of a simple low-cost model, amenable, that surpasses the complexity of models that emulate the central nervous system.

Chapter I

Literature Review

Introduction

1. The neurodegenerative disorders paradigm

Neurodegenerative disorders are characterized by progressive loss of specific neuronal populations leading to a decline in brain functions. These pathologies are responsible for substantial medical and public health burdens with millions of people being diagnosed worldwide [1]. Currently, neurodegenerative diseases are not curable, and treatment focuses on the relieving of symptoms and delay disease progression.

The incidence of neurodegenerative disorders increases dramatically with age. Particularly in Europe and parts of Asia where, on average, the populations are older the challenges with neurodegenerative diseases, which includes Alzheimer's and Parkinson's disease and amyotrophic lateral sclerosis, are daunting [2].

Alzheimer's disease (AD) is the most common neurodegenerative disease with a prevalence of nearly 30% among people ≤ 85 years [1]. The primary clinical manifestation of AD is an accelerated loss of cognitive function leading to dementia in the majority of the cases [1]. The two major pathological hallmarks of AD are the formation of protein aggregates amyloid-beta ($A\beta$), in the form of extracellular plaques. Alterations in the microtubule-associated protein Tau, such as hyperphosphorylation and missorting, which aggregates in neurofibrillary tangles are also a common feature of AD [8, 9]. Although genetic and *in vivo* studies suggest that abnormal production of $A\beta$ is upstream of Tau aggregation, the connection of these two hallmarks is not well understood [8, 10]. Nonetheless, misfolding of Tau correlates with the loss and destabilization of axonal microtubules (MT), and axonal transport alterations, which ultimately results in neuronal degeneration [8-10].

Parkinson's disease (PD) is the second most common neurodegenerative disease, characterized by a significant loss of dopaminergic neurons in the substantia nigra, and whose clinical features are rest tremor, rigidity, and postural instability [1, 11, 12]. The prevalence of PD may reach 2% in people ≤ 65 years but is relatively rare below 50 [1]. The pathological hallmark of PD is the Lewy body inclusions that are composed of α -synuclein, a protein involved in synaptic function [9, 13]. As in AD, there are evidence of MT dysfunction in PD. *In vitro* studies with cell lines overexpressing α -synuclein and analysis post-mortem of patient's brains reveal a link between PD pathology and MT alteration, including decreased MT networks, MT-dependent trafficking leading to axonal transport disruption, and degeneration [5, 14, 15].

Amyotrophic lateral sclerosis (ALS) is a motor neurodegeneration disorder, described by a progressive muscle disability as a consequence of neuronal cell death. As seen in the other neurodegenerative diseases, ALS incidence rises at older ages [1, 16]. Various observations support the hypothesis that deficits in axonal transport and hyperdynamic MTs may be related to the selective vulnerability of motor neurons in ALS since certain motor neurons have the longest axonal projections of neurons in the body [5, 17, 18].

As evidence here, many neurodegenerative diseases are correlated with dysfunctional axonal transport, a process supported by MTs. Although abnormalities vary among different neurodegenerative disorders, there are evidence that the transport deficiencies result from

decreased axonal MT stability [5, 6, 9]. Since neurons are very susceptible to MTs regulation, it is not surprising that MT dysfunctions may contribute to the onset of neurodegenerative disorders [6].

2. Microtubules in neurodegenerative disorders

1. Microtubule structure and function

MTs constitute the main component of the eukaryotic cytoskeleton, an integrated filamentous network that provides most of the cell spatial organization and it's responsible for their shape, motility, and regulates diverse cell functions such as intracellular trafficking and cell division [6, 19, 20]. Among the cell components, MTs are one of the most complex structures.

MTs are composed of 2 building blocks: α -tubulin and β -tubulin that form heterodimers (Fig. 1) [3, 20]. It assembles head-to-tail to form protofilaments and associate laterally to create a hollow tube with an outer diameter of approximately 25 nm [20, 21]. The majority of eukaryotic cells form MTs with 13 protofilaments [20, 22].

Both MTs and the protofilaments are intrinsically polarized due to the presence of two distinguished ends: a minus (-) end that exposes an α -tubulin subunit and the opposite plus (+) end that exposes an β -tubulin subunit, exhibiting a structural and functional polarity (Fig. 1) [6, 20, 21].

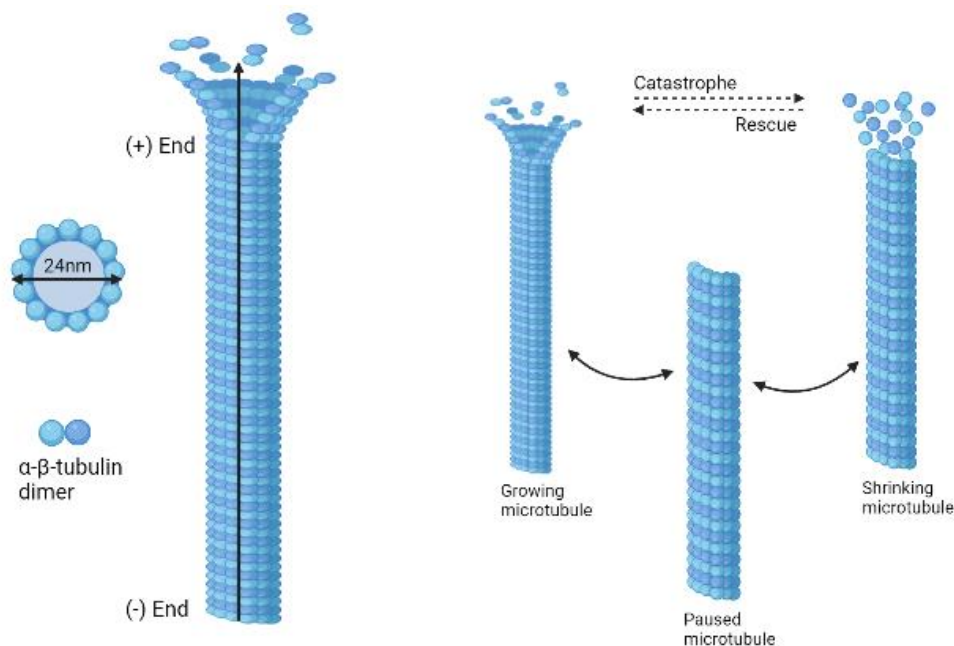


Figure 1. Microtubule main components and their dynamic behaviour. Created in BioRender.com

A key property of MT is its dynamic behaviour. MTs are constantly suffering cycles of assembly and disassembly, and the co-existence of these two stages in the same environment is known as “dynamic instability” [21, 23]. MT assemble is favoured at the plus end which occurs through the addition of an α/β heterodimer with each subunit containing a bound to a GTP molecule [20, 24]. After the addition to the MT, the GTP is hydrolysed to GDP, and prolonged exposure of this GDP in the plus end can lead to MT depolymerization [20, 23, 24]. Thus, stable growth is believed to depend on the presence of a cap of GTP-tubulin at the plus end. Conversion from

growth to shrinkage is known as “catastrophe”, and the opposite is called “rescue”. The dynamic process is crucial to MTs role in cells functions (Fig. 1) [23].

2.2 . Microtubules organisation in neurons

Connected networks are formed by neurons that process and transmit electrochemical information. A classic vertebrate neuron consists of a small soma, an axon specialized to transmit information, and several dendrites that form a receptive field for incoming information [25-27]. To maintain and establish their unique morphology and functionality neurons are highly polarized cells, being the MTs one of the central pieces in their dynamics together with actin filaments.

MTs are crucial for neuronal early developmental stages, such as migration of the soma, and orientation of the growth cone at the tip of the elongating axon (Fig. 2) [4, 27, 28]. Nevertheless, MTs are also important throughout the life of the neuron, to maintain proper morphology, accommodate changes that correspond to cognitive plasticity, and enable axonal and dendritic transport [4, 27, 28].

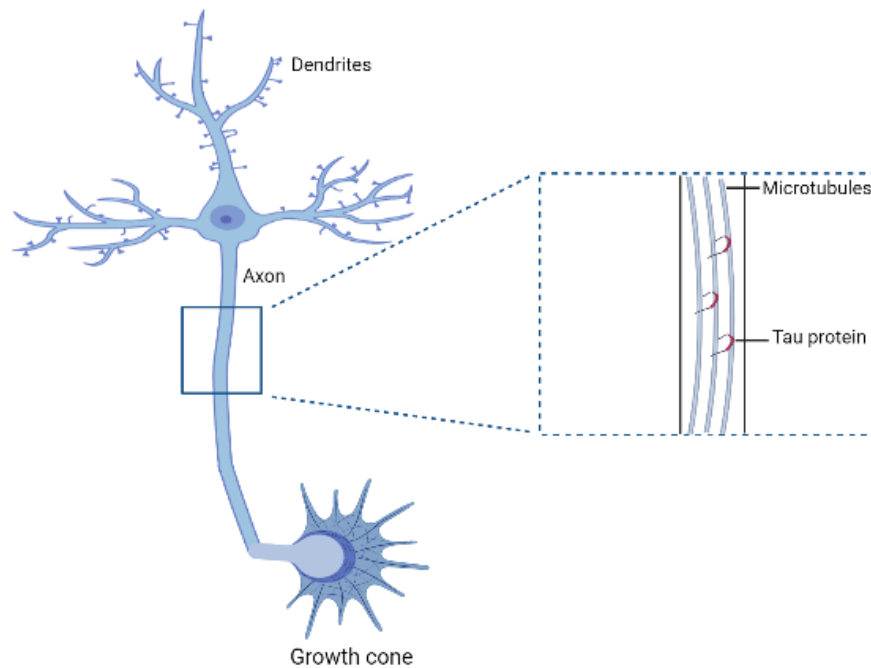


Figure 2. Microtubule organization in neurons. Created in BioRender.com

Thus, neurons have a dense MT network that consists of individual MT with different lengths, orientations, and degrees of stability [3, 29, 30]. The maintenance of the critical microtubule architecture requires tight control over these parameters.

MTs arrays are heterogeneous. While some are only a few micrometers, others can exceed a hundred micrometers in length. The different sizes of the MTs correspond to different functions in neurons. Long MTs have a dual function, acting as a structural backbone and establish tracks for long-distance organelles transport along the neurons, while short MTs are mobile elements providing high dynamics and means by which tubulins are actively transported around axon and dendrites [27, 31].

A specificity of neuronal MTs is that unlike most cell types where MTs are nucleated at a microtubule-organizing center (MTOC), in mature neurons, most MTs do not emerge from the MTOC [3]. In young neurons, the release of MTs from the MTOC is important for axonal growth. However, over time this activity is almost lost, which leads to the variation in the MTs orientations detected in mature neurons [6]. While in axons MTs have a uniform orientation, with plus ends out, in dendrites, microtubules have mixed orientations [28, 32, 33].

Given that MTs are the substrates for molecular motor proteins, responsible for motor-driven forces that transport cargoes toward the plus or minus end of the MTs, the polarity patterns established in the neurons are partially regulated by motor proteins [25, 34, 35]. For example, the cytoplasmic dynein is the main motor protein for transporting minus-end MTs into both axons and dendrites, while kinesin-6/12 are responsible for transporting the plus-end MTs only into dendrites [4, 25, 35].

The axonal projections can reach up to 1 meter in length, making axonal transport critical to the health and viability of neurons, given the necessity of transport organelles to and from the axon terminus [5]. Presumably, because of the specialized role that MTs play in axonal transport it is believed that the targeting of certain motor proteins to specific MTs is associated with the encoded neuronal MTs network. In neurons, MTs are characterized by a lower degree of dynamic instability comparing with non-neuronal cells, [5] and these dynamics are tightly regulated by different tubulin isotypes, post-translational modifications (PTMs), and binding of diverse MT-associated proteins (MAPs), a set known as “the tubulin code”.

2.3. Microtubule regulation

The MTs patterns start with the generation of expression of different α - and β -tubulin isotypes, a concept known as the “multi tubulin hypothesis” [3, 36]. Each tubulin is expressed by multiple genes at different loci, which is believed to have diverged evolutionarily from a single gene. TUBB2A, TUBB2B, and TUBA1A, TUBA1B, TUBA1C are examples of clusters in the genome and are likely the products of genomic duplications [37]. It is important to consider that while general tubulin isotypes are highly conserved between different species, less common and more function-specific isotypes appear to have evolved as novel MTs arose through divergent evolution (Table 1) [38].

Table 1. Tubulin isotypes expressed in the brain.

Human Gene	Tubulin Isotype	References
TUBB	class I β -tubulin	[6]
TUBB3	β 3-tubulin	[39]
TUBA1A	α -tubulin1A	[6, 40]
TUBA8	α 8-tubulin	[6, 41]
TUBB2B	β -tubulin2B	[6]
TUBA2A	β -tubulin2A	[6]
TUBA4A	α -tubulin4A	[6]
TUBA4B	β -tubulin4B	[6]

The tubulin isotypes differ only by a few amino acids, and these minor differences are enough to alter the MTs properties by affecting the MT dynamic [38].

In neurons, the MTs are composed of specific tubulin isotypes. β_3 -tubulin and β_2 -tubulin are the predominant tubulin isotypes in neurons, and β_3 -tubulin is exclusively found in neurons and in Sertoli cells [27, 38, 42, 43].

Another important regulatory mechanism is post-translational modifications (PTMs) (Table 2). Beyond the existence of tubulin isotypes, these are further diversified through many chemically induced PTMs [44]. Not surprisingly, long-lived MTs accumulate a greater number of PTMs.

Almost all these modifications such as acetylation, tyrosination, polyglutamylation, phosphorylation, methylation, and ubiquitylation are reversible and take place on the carboxy-terminal domain of tubulins, except acetylation that occurs on the luminal surface of MTs [3, 38, 45].

Neuronal MTs are enriched in a diversity of PTMs (Table 2), and many modifications correlate with specific parts of the neurons [3, 6]. The widely accepted hypothesis points to the idea that PTMs differ between neuronal compartments and change during neuronal differentiation [26, 38]. Therefore, specific patterns of PTMs are accumulated at specific stages of neuronal differentiation and maturation, locally controlling the MTs functions. For example, polyaminated tubulin is rare in developing neurons, but is a prominent PTM in adult neurons [4].

Table 2. Post-translation modifications (PTMs): acetylation, methylation, detyrosination, glutamylation or polyglutamylation, polyamination and phosphorylation.

Post-translational modifications (PTMs)	Modification site	Enzymes catalysing PTM	References
Acetylation	α -tubulin Lys40	α -tubulin acetyltransferase	[46-49]
	β -tubulin Lys 252	San acetyltransferase	[50]
Methylation	α -tubulin Lys40	SET domain-containing protein 2	[51]
Detyrosination	α -tubulin C-terminal Tyr residue	Vasohibin proteins in complex with small vasohibin-binding proteins	[38, 52, 53]
Glutamylation or polyglutamylation	α -tubulin and β -tubulin C-terminal tails	Tubulin-tyrosine ligase-like proteins	[54-56]
Polyamination	β -tubulin Gln15	Transglutaminases	[57]
Phosphorylation	α -tubulin and β -tubulin Tyr residues	Neuronal proto-oncogene tyrosine-protein kinase SRC	[58, 59]
	β -tubulin Ser172	Cyclin-dependent kinase 1	[60]
	β -tubulin Ser172	Dual-specificity tyrosine-regulated kinases	[61]
	β_3 -tubulin Se444	Unknown	[62]

Part of the MTs regulation mediated by PTMs is associated with the predisposition of microtubule-associated proteins (MAPs) to bind to these specific parts of the MTs.

MAPs constitute a class of heterogeneous proteins that perform varied functions as regulators of MTs, such as modulation of MTs stability and dynamics [38]. Therefore, MAPs can be divided into five groups: i) motile MAPs; ii) MT end-tracking proteins (TIPs); iii) MT-severing enzymes; iv) MT nucleators; v) structural MAPs (Table 3) [6].

In neurons, the MTs appear to be governed through interactions with MAPs, where several specific MAPs have been found (Fig. 3) [5, 63]. Indeed, the rapid turnover observed in axonal and dendritic MTs is believed to be less dynamic due to their interactions with a specific subclass of MAPs [64, 65].

Table 3. Microtubule-associated proteins (MAPs) and their binding sites in microtubules.

Microtubule associated protein (MAP)	Binding site	References
CAMSAP family	MT minus ends	[3, 4, 66, 67]
Tau	MT lattice	[4, 6, 68, 69]
MAP2	MT lattice	[6, 68-70]
MAP1b	MT lattice	[4, 6, 69]
Doublecortin	MT lattice	[4, 69]

2.4. Microtubule role in neurodegenerative disorders

Neuronal MTs organization is tightly regulated. Hence, considering that these mechanisms act synergistically and not separated, it is not surprising that abnormalities in any of these key pieces can trigger a cascade of events leading to MTs dysfunction in neurons.

The damage of MTs functions is considered one of the central physiopathological mechanisms of several neurodegenerative disorders [6]. The evidence of MTs direct role in neurodegenerative diseases comes from the observations that MTs mass is commonly diminished, MTs dynamic and stability is altered, and that there is potential to the corruption of the MTs polarity patterns and MTs-mediated transport [4, 6].

Specific mutations in genes encoding tubulins, dysfunctions of MAPs, and alterations in the levels of tubulin PTMs are linked with different forms of neurodegenerative diseases. Indeed, these alterations on MTs have been observed in AD, PD, ALS, Hereditary Spastic Paraplegia (HSP), and others [4, 6, 71].

Thus far, four tubulin isotypes were associated with neurodegenerative disorders, TUBB3, TUBB2A, TUBA4A, and TUBB4A (Table 4) [6, 72-78]. For example, TUBA4A is the highest β -tubulin expressed in the human motor cortex, and brain expression levels have been reported to dramatically increased with age [79]. Thus, this pattern coupled with mutations in TUBA4A may explain why these dysfunctions can cause ALS, a late-onset disease characterized by motor neurodegeneration [74].

Table 4. Tubulin isotypes altered during neurodegenerative disorders.

Human Gene	Amino Acid Mutation	Associated Disorder	References
TUBA4A	W407X	Amyotrophic Lateral Sclerosis	[74]
	G43V		
	R215C		
	R320C		
	R320H		
	A388T		
TUBB2A	D417N	Sensory Motor Polyneuropathy	[80]
TUBB3	D417N		[72, 73]
	D417H		[72]
	E410K		
TUBB2B	E421K	Polymicrogyria	[81]
	E288K	Pachygyria	[77]
TUBB4A	H190Y	Congenital-onset spastic paraplegia and mild hypomyelination	[75, 82]
TUBB4A	D353V	Early-onset progressive spastic paraplegia	[76]

Besides tubulin genes, several findings reveal that alterations in PTMs patterns are also implicated in neurodegenerative disorders (Table 5) [6]. Reduced levels of acetylated α -tubulin have been described in the neurons of patients affected by AD and Hunting disease (HD) [83].

Table 5. Altered post-translational modifications (PTMs) associated with neurodegenerative disorders.

Post-translational modification (MAP)	Abnormality	Disorder	References
Acetylation	Elimination by mutated K40 or deleted gene encoding α TAT1	Touch sensitivity	[84]
	Reduced levels	Alzheimer's disease	[6]
		Charcot-Marie-Tooth disease	
		Hunting disease	
Glutamylolation	Hyper-	Neurodegeneration in general	[85]

However, the MT loss observed in cases of neurodegenerative disorders is best documented in diseases called “tauopathies” [4, 5, 86-88]. In these disorders, the MAP tau dissociates from MT due to hyperphosphorylation, which promotes tau sequestration into neuronal inclusion and reduces MT stability [89-92].

In wild-type conditions, a MT stabilizer MAP protein (e.g. tau) alters a labile into a stable domain [27]. However, in neurodegenerative conditions, MTs destabilize, eventually disintegrating, as they lose tau. Indeed, several studies point out the idea that oligomeric $A\beta$ accumulation, a hallmark of AD, cause tau mislocalization, which is correlated with the loss of MTs [8, 93, 94].

In some tauopathies, such as frontotemporal dementia and AD, the abnormal phosphorylation of tau is frequently associated with mutations in MAPT, the tau encoding gene [86-88, 95]. In AD patients' brains, more than 40 phosphorylated sites (Table 6) were detected in tau protein, which depending on their position, seem to differently reduce tau affinity for MTs [96].

In addition other MAPs are also important regulators for MT dynamics, such as parkin, spastin (Table 6) [6, 8, 97]. Their loss-of-function is also associated with different neurodegenerative disorders.

In the case of PD, parkin is the protein that is reported to be responsible for MT dysfunction. Parkin is an E3 ubiquitin ligase that binds to α - β heterodimers, enhancing the ubiquitination and degradation of tubulins [98-100]. Thus, mutations in the gene PRKN, responsible for encoding parkin protein, are the most frequent cause of autosomal recessive juvenile parkinsonism, and a great part of the contribution to the development of familial and sporadic early-onset PD (Table 6) [98, 99].

In contrast to tau, spastin main function is to disrupt the MTs by destabilizing tubulin-tubulin interactions (Table 6) [8, 101]. Mutations in spastin protein reduces the disrupted MTs and is mislocalized on axonal MT, which secondarily causes mislocalization of mitochondria due to associated defects in axonal transport [9, 102]. Accordingly, point mutations and deletions of SPAST, the gene encoding spastin, are linked to approximately 40% of autosomal dominant forms of Hereditary spastic paraplegia (HSP) [101].

Table 6. Abnormalities in microtubule-associated proteins (MAPs) related to neurodegenerative disorders.

Protein	Abnormality	Disorder	References
Tau	Phosphorylated Ser199	Alzheimer's disease	[96]
	Phosphorylated Ser202		
	Phosphorylated Ser235		
	Phosphorylated Ser262		
	Phosphorylated Ser356		
	Phosphorylated Ser422		
	Phosphorylated Thr212		
	Phosphorylated Thr231		
	Mutations in the gene MAPT	Tauopathies	[9]
	Hyperphosphorylation		
Parkin	Mutations in the gene PRKN	Parkinson's disease	[103]
α -syn	Missense mutation, duplication and triplication of the gene SNCA		
LRRK2	Mutation in the gene		
APP	Mutations and duplications in the gene	Alzheimer's disease	[9]
Presenilin	Mutations in the gene		
Spastin	Point mutations in the gene SPAST	Hereditary Spastic Paraplegia	
	Large deletions in the gene SPAST		
SOD1	Mutations in the gene SOD1	Amyotrophic lateral Sclerosis	[18]

Lastly, given the importance of MTs in axonal transport mentioned previously, it is not surprising that also the defective axonal transport emerged as a commons factor in neurodegenerative disorders. The notion of impaired axonal transport in several neurodegenerative conditions is given by numerous studies where the progressive accumulation of specific proteins in different types of neurons was detected [5, 9].

Furthermore, studies in post-mortem PD patient brains concluded that kinesin and dynein levels, proteins responsible for motor-driven in MT, are decreased in cases of sporadic forms of PD, with the levels of kinesin starting to be altered even before the dopaminergic loss [15]. This

analysis supports the notion that axonal transport alterations are associated with neurodegeneration observed in PD pathology.

In cases of HSP, it was detected mutations in genes that encode proteins involved in axonal transport, highlighting the relevance of the process in long motor neuron axons [102]. Thus, defective intracellular transport can directly trigger motor neuron degeneration.

Although the causes of deficient axonal transport may vary among the different neurodegenerative disorders, it appears that the transport abnormalities result from a reduction of axonal MT stability.

2.5. The role of microtubule-stabilizing drugs

It is unknown whether MTs alterations in neurodegenerative diseases are a primary or secondary factor in the degeneration process. Regardless, the use of microtubule-stabilizing agents is emerging and a potential treatment for neurodegenerative/neurological diseases [6, 104-106].

Microtubules-stabilizing agents are among the most clinically use chemotherapeutic drugs, allowing to redirect the knowledge gained in the cancer therapeutics and apply in neurodegenerative diseases [106, 107]. These drugs act by stabilizing MTs and altering the mitotic spindle during cell division, inhibiting their proliferation [5, 106].

The first proof-of-concept was paclitaxel, a first-line anticancer drug, in 1999 [108, 109]. Previously, it was established that this compound counteracts the dynamic instability of MTs in cancer cells by binding within the lumen of the MT at a site in the β -tubulin [110].

To test the hypothesis of microtubule-stabilizing agent role in the therapy of neurodegenerative disorders, a Tau transgenic (Tg) mouse model was treated with a version of paclitaxel [111]. Indeed, it was observed an improvement in MT density, an increased axonal transport in spinal motor neurons and, at the same time, an enhanced motor performance [108, 111].

However, paclitaxel is an unfit therapeutic candidate for neurodegenerative diseases due to some raised concerns, inability to cross the BBB resulting in limited brain bioavailability, poor water solubility, susceptibility to P-glycoprotein (P-gp, a protein present in the cell membrane involved in the efflux of foreign compounds) and high toxicity related with serious adverse effects [112, 113]. The therapeutic effect of paclitaxel in the Tau Tg mouse model was obtained for its absorption in neuromuscular junctions since in the animal model tested the tau pathology is mostly confined to the spinal cord and brain stem. Although the BBB permeability could be bypassed with different strategies such as nanoparticles, chemical modifications, and focused ultrasound, the other characteristic of the Taxol are still limiting factors [106, 111, 114, 115].

More recently, studies were conducted to evaluate the potential therapeutic effect of epothilone D (EpoD), a brain-penetrant MT-stabilizing drug. EpoD is a taxol-derived compound that prevents MT disassembly by interacting and stabilizing β -tubulin [7, 109]. This molecule is one of the most promising MT stabilizers in the neurodegeneration field due to its excellent BBB penetration and consequently prolonged brain retention, which allows a selective accumulation in the central nervous system [7, 111].

As well paclitaxel, EpoD was used in the treatment of a Tau Tg mouse model in several studies [7, 109, 111]. These revealed an improvement in MTs density and axonal transport while reducing axonal dystrophy. EpoD also enhanced cognitive performance and decreased hippocampal neuronal loss, giving evidence of attenuated neuropathology in this AD-like mouse model [7, 109]. The same encouraging results were obtained when treating other animal models recapitulating other neurodegenerative disorders, such as PD, ALS, and HSP and even following brain traumatic and spinal cord injuries [106, 109, 113]. Cappelletti *et al.* demonstrated the neuroprotective effect of EpoD in PD-like mouse model induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), where daily administration of the microtubule-stabilizing drug rescue MT defects [116]. Furthermore, in *in vitro* approaches, neuronal primary cultures from amyloid precursor protein-mice, EpoD treatment showed an increased dendritic spine density [117].

Since EpoD treatments showed promising results in different neurodegenerative-like pathologies animal models and in *in vitro* studies, phase I clinical trials were conducted to assess the safety, tolerability, and effect of low doses of EpoD in patients with AD [118]. However, translational into clinical was not successful, with EpoD clinical trials being interrupted after phase I. The results were never published, and the causes for the suspension of the trial are unknown. Nevertheless, a new clinical trial with the same objectives started, it was already completed in September 2019, and the results are expected soon [119, 120].

In the meantime, other MT-stabilizing agents are being investigated as promising therapeutic compounds for neurodegenerative disorders (Table 7).

Although MT-stabilizing compounds are regularly used for anticancer therapies they have been shown to cause unwanted side effects in off-target parts of the body. However, as a therapy in neurodegenerative diseases, only low doses of MT-stabilizing agents are required to compensate for the MT abnormalities without substantially affecting cells in the periphery [5, 121]. For example, studies with EpoD revealed that approximately 1/30 of doses employed in cancer clinical trials are required to achieve MT-stabilization in the diseased brains [5].

Another critical challenge is the maintenance of the MT-stabilizing agent in the nervous system to allow prolonged drug activity, a feature undesired in the majority of other diseases. Thereby, the studies are towards a compound with prolonged brain retention, which results in clearance of the MT-stabilizing agent from the periphery minimizing side effects and boosting drug effect with low drug doses [4, 5, 122].

Nevertheless, one should consider that the cytoskeleton component tubulin is present in cells throughout the body. Since MT-destabilization is needed for cell division it is expected that MT-stabilizing drugs might interfere with the normal regulation of the organisms. So, secondary effects must be evaluated.

Table 7. Examples of microtubule-stabilizing agents in the field of neurodegenerative disorders.

Drug	Effects	Model(s)	Stage of development	Reference
Paclitaxel	Restores axonal transport in spinal axons, reduces the motor phenotype, increases the level of stable detyrosinated tubulin and the number of MTs	PrP T44 mouse, Tau Tg mouse	Interrupted (unable to cross BBB)	[111, 117]
Clinical trials (Phase I)				
Epothilone D	Reverses behavioural and cognitive deficits, clears tau pathologies, reduces neuron loss, restores the levels of acetylated α -tubulin and increases dendritic spine density	Tau-PS19 mouse, iPSCs derived from HSP patients	NCT01492374 (interrupted, unknown results) NCT01966666 (pending results)	[109, 118, 120, 123]
CNDR-51657	Improves MT density in hippocampal neurons and reduces axonal dystrophy which leads to reduction in tau pathology	Tau-PS19 mice	Pre-clinical development	[124]
Noscapine	Attenuates the abnormal axonal transport and improves MT stability	SOD1-mutant mice	Pre-clinical development	[125]
Davunetide (NAP)	May prevent MT disruption, enhances axonal transport and improve cognitive performance	AD-mouse model, ALS-mouse model	Clinical trials (Phase II) NCT 01056965	[126-128]
Cabazitaxel	Disrupts cell cycle by its effects on the MTs	Primary neurons	<i>In vitro</i> studies	[106, 129, 130]
Discodermolide	Stabilizes MTs	Cell lines	<i>In vitro</i> studies	[131]
Dictyostatin	Increases levels of acetylated tubulin, indicating that it increases MT stability	CD-1 Mice	Pre-clinical development	[132]

The advances in MT-stabilizing agents might offer new therapeutic paradigms to intervene and ultimately treat neurodegenerative diseases. The data presented support the potential of MT-stabilizing drugs as a therapeutic in AD, PD, ALS, and others by rescue MTs dysfunctions and/or axonal transport abnormalities.

The results lead to believe that in the near future MT-stabilizing agents will be an important part of neurodegenerative treatments. However, for more preclinical approaches to translate to the clinic, there is a need to identify additional MT-stabilizing drugs with prolonged brain retention and low required doses for a microtubule-stabilizing effect, candidates that can properly balance efficacy and safety.

3. Drug repurposing towards the treatment of neurodegenerative disorders

The market for disease-modifying drugs in neurodegenerative disorders not only is colossal but is also increasing rapidly [2]. Considering the case of AD, it is estimated that 40 million people were diagnosed with the disease in 2015 worldwide, and this number is expected to rise to 100

million by 2050 [133, 134]. However, despite the efforts pursuing the discovery and development of therapeutics, the outcomes are limited. A disease-modifying drug is still being pursued, and the treatments currently rely on symptoms relief [135, 136].

Neurodegenerative disorders posed a significant challenge to drug discovery over the past century, with traditional drug discovery (Fig. 3) fallen short since almost no drugs enter the market [137, 138]. But why is this happening?

In the last years, there has been a high degree of failure in several clinical trials for drugs in neurodegenerative disorders. A major factor contributing to this is the difficulty of translating findings from pre-clinical drug testing (traditionally animal-based models, mostly mouse) to human patients [138-140].

Thereby, joining the recent failures in many clinical trials targeting neurodegenerative diseases, and the ethical concerns in animal experiments, there have been doubts about the capability of animal models to recapitulate neurodegenerative pathology [2, 141-143]. So, there is a demand for more capable research tools in this field, namely in drug responsiveness.

Furthermore, the traditional drug development approaches are expensive and lengthy (Fig.3) This pathway entails extensive research with discovery and preclinical stages, clinical trials, regulatory authorities review, approval, and post-market monitoring, which can take 13-15 years and over US \$1 billion [144, 145]. Even after all this process it is estimated that only 10% of compounds reach the market [146].

Nevertheless, as mentioned already, the number of patients living with neurodegenerative disorders is rapidly increasing and already exerts high pressure in the healthcare systems worldwide [147]. The demand for an effective drug is enormous. So, it is necessary to apply alternative strategies for reducing the drug discovery time frame and accelerate the development of alternative therapeutics for neurodegenerative diseases.

Novel drugs came from three main sources: natural products, synthetic synthesis, and already existing drug compounds [136]. This last source, known as drug repurposing, has recently seen several initiatives driven by academic and non-profit organizations complementing the work of pharmaceutical companies in seeking new therapeutic areas for approved drugs [148]. Indeed, approximately 30% of compounds after phenotypic screens targeting pharmacological activity have an indication to an alternative therapeutic use [149].

Drug repurposing is “the application of established drugs to new therapeutic indications”, offering a cheaper and more rapid drug development route (Fig. 3) [137]. Specifically, in diseases with an unmet clinical need, such as neurodegenerative disorders, there has been an increase in pursuing drug repurposing.

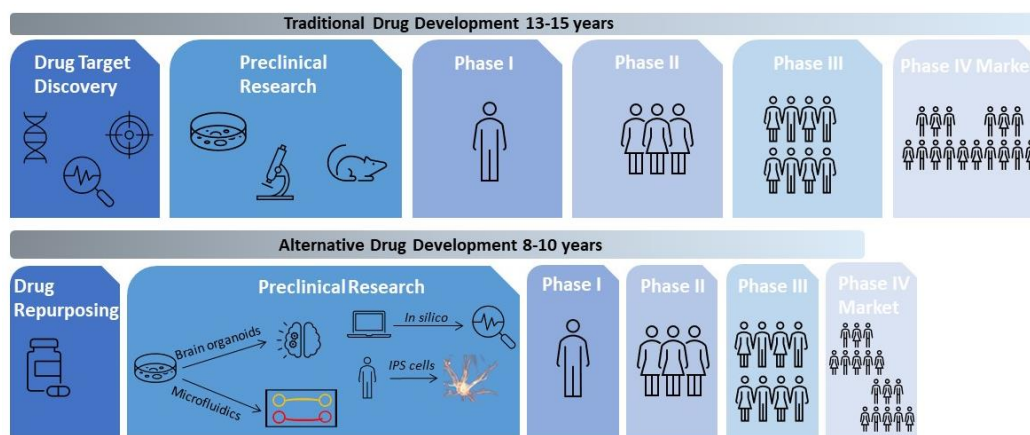


Figure 3. Traditional drug development vs alternative drug development (starting with drug repurposing) [144, 150].

The exploration of drug repurposing provides an attractive alternative to fill the gap between the increasing incidence of neurodegenerative diseases and the long-term duration of traditional drug development. This alternative approach bypasses some steps in drug development, reducing the time frame in 5-7 years [150].

Since drug repurposing focuses mainly on testing drugs approved by the regulatory agencies, this strategy has the advantage of identifying compounds that meet traditional pharmacocriteria since they have already been optimized for these features [137, 149]. Thus, drug repurposing allows to substantially lower the costs of development of a drug since the safety and tolerability profiles are already established, which may require smaller and more cost-effective clinical trials [136, 139, 151].

The method of drug repurposing, despite the 30% success rates, has its challenges [146, 152]. First, limited patent protection often means a lack of commercial interest or incentive by pharmaceutical companies [146]. Second, the *in vitro* evaluation of the candidate's drugs is still limited by traditional and improper cell- and animal-based models that have posed meaningful challenges in drug discovery against neurodegenerative disorders. So, the choice of the most relevant model is key for a successful drug development.

Screening of compounds for repurposing means involves mainly three approaches: *in silico* screening, target-based assays, and phenotypic screens [136, 149, 152].

In silico assays are computational approaches that base the outputs on analysing the compound molecular structure, combination of drug-target interactions, networking of genes, and common adverse effects, identifying more potential opportunities for repurposing [146]. Target-based assays use techniques that enable the identification of diverse compounds that bind to a specific target involved in a specific disease [152, 153]. Lastly, phenotypic screening allows the identification of novel drugs based on changes in a specific-relevant phenotype of *in vitro* or *in vivo* models [148, 152].

The strengths of the phenotypic screening may particularly stand out for drug repurposing in cases where target validation approaches have had little success, diseases where underlying mechanisms are not yet clear [148, 154]. As already stated, these are the challenges presented for drug development in neurodegenerative disorders. Therefore, phenotypic screening does

not require a complete understanding of the mechanisms behind neurodegeneration, and since it searches for alteration in disease-phenotype, the repurposed “hits” might be translated more directly into a novel therapeutic [154].

For now, there is a need for the development of an unbiased and rapidly phenotypic screening platform applicable for neurodegenerative diseases drug screening. In the future from the data generated new potential drugs can enter clinical trials.

3.1. High-throughput screening as a powerful tool for drug discovery

To study and acquire neuron MT-stabilization phenotypes two main strategies can be followed to discover potential MT-stabilizing compounds: the candidate drug approach and the high-throughput screening (HTS) [155, 156]. The candidate drug approach is leaning to situations where there are indications for a specific pathway responsible for a certain phenotype and, so, a restricted and well-defined group of compounds is screened [155]. By contrast, HTS allows testing hundreds of thousands of compounds per project and does not require wide knowledge in specific pathways and molecular mechanisms, which as previously stated, suits the neurodegenerative disorders area [155, 157]. These two main strategies are equally valid. However, given their particularities the use of the HTS strategy to pursue MT-stabilizing agents suitable for neurodegenerative diseases could be highly rewarding. But why is HTS such a powerful tool, and what impact can have in the initial steps of drug discovery of MT-stabilizing agents for neurodegenerative disorders?

Identifying potential therapeutics could take years when using conventional “trial and error” methods, but HTS is an iterative assay that allows scientists to screen compounds in a relevant model for the disease, hence identifying potential candidates/ “hits” [157, 158]. HTS relevance in drug discovery arises from the need of screening large libraries of compounds in a fast, cheap, and with efficient protocols. Indeed, one viable drug can emerge from millions of screened compounds, causing the elimination of a significant fraction of hits, thus allowing to focus on the more physiological relevant candidates.

HTS came to light in the process of discovery in pharmaceutical and biotech companies but is now also well-established for research in academia [159, 160]. HTS has evolved in the last 15-20 years, being optimized along with the triangle principles of HTS: time, cost, and quality of the process (Fig. 4) [160]. In the beginning, the HTS protocols were performed in a 96-well plate format, but the requirement of readiness evolved the format for 1536-well microplates, which accommodates 4 times more samples (Fig. 4) [159, 160]. Thus, HTS is evolving into a key strategy of modern drug discovery.

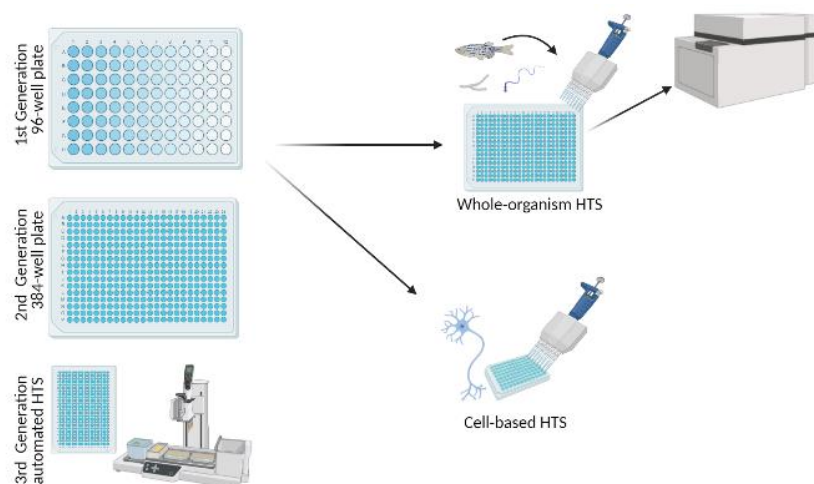


Figure 4. High-throughput screening (HTS) evolution. Created in BioRender.com

Classically, HTS uses cell-based technologies (e.g. immortalized cell lines or rodent primary cultures), and the detection is based on measurements of molecular interactions by fluorescence, luminescence, or absorbance readouts [157, 161-163]. However, cell lines have altered parameters such as being immortalized, lacking physiological relevance, and in rodent primary cultures there is the lack of scalability needed for HTS. Moreover, the hits generated during HTS require further assays in cells and/or animals, and most of them fail after these biochemical assays [155, 163, 164].

To bypass these adversities, has been pursued the possibility of carrying out high-throughput *in vivo* screening. The more complex HTS utilize non-mammalian species, more commonly the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, and the fish *Danio rerio* [164-167]. These organisms have in common genetic amenability, low cost, easy culture conditions, and miniaturization characteristic, all compatible with HTS. The foremost advantage is enabling HTS in a whole-organism context, most relevant in the case of neurodegenerative disorders where cell-based technologies are insufficient in mimicking the disorder and the complexity of the brain.

There are two strategies for HTS in whole-organisms [164]. The first consists of testing chemical libraries on wild-type organisms to evaluate the ability of the compounds to induce a specific phenotype as MT-stabilization, morphological defects, slow growth, among others. The second approach is also based on a phenotypic screen but consists of testing chemical libraries against an abnormal phenotype to evaluate the capacity of the compounds to reverse to a wild-type phenotype [164].

HTS in whole-organisms has the capacity to, up to some point, select potential drugs with an improved safety profile earlier in the drug discovery process and by screening based on functional phenotypes [164]. This strategy allows reducing both costs and time, enabling a more simple, rapid, and promising progress towards the development of new therapeutics for neurodegenerative disorders.

Indeed, non-mammalian models have successfully been part of drug discovery for neurodegenerative diseases and, in combination with HTS, are a potential tool for the discovery of novel MT-stabilizing agents [164, 168-170].

For the success of the HTS, it must be defined a relevant model for the project. There are two main adversities regarding the discovery of promising “hits” in the neurodegenerative field: the relevance of the models used and the limitation in term of quality and size of the compound library available to HTS [157].

For the discovery of promising MT-stabilizing compounds, it should be selected a whole-organism model where cytoskeleton MTs are similar to the morphology demonstrated in human neuronal cells. Furthermore, the model used must be compatible with HTS and generate comparable, homogeneous, and reliable data [164].

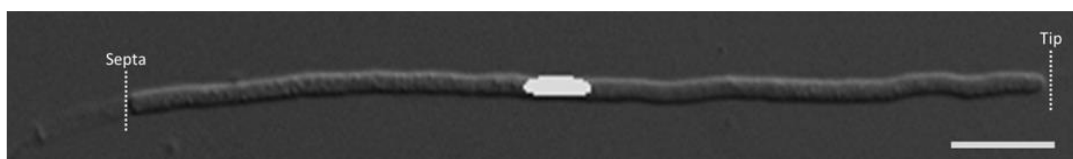
4. *Ustilago maydis*: a fungi drug screening platform for neurodegenerative disorders

The fungus dimorphic basidiomycete *Ustilago maydis* (Fig. 5) is a plant pathogen responsible for corn smut disease, belonging to the order Basidiomycota and family *Ustilaginomycetes* [171, 172].

U. maydis presents one of the smallest sequenced and accessible genomes in the pathogen group (≈ 20.5 Mb, with more than 6500 genes), which propel multiple laboratories to establish a range of genetic, bioinformatics and molecular biology techniques to this fungus [172, 173].

One of the most remarkable aspects of this fungus is that despite growing as a yeast-like cell (Figure 5), to initiate pathogenic development or under stress conditions (e.g. nutrient starvation and pH alterations), *U. maydis* can switch to filamentous growth (Fig. 5) [172-174]. The filamentous state form (also known as hyphae) is characterized by elongated hypha cells that expand by polar tip growth and features a central nucleus [173, 175, 176].

Hyphae



Yeast-like

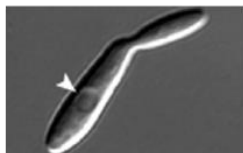


Figure 5. *Ustilago maydis* dimorphic: yeast-like and hyphae forms.

Curiously, genome-wide analysis reveals that *U. maydis* is more closely related to humans than other non-mammalian species normally used in HTS, and numerous proteins are present only in *U. maydis* and *Homo sapiens* [172, 173]. Indeed, studies have demonstrated that fungi are

evolutionary related with mammals, which makes the basic principles of cellular processes conserved between the two species [173].

For several reasons, *U. maydis* has been considered a well-suited model for cellular mechanisms. The life cycle of this fungus can be completed in a few days, allowing the production of large numbers of mutants with a range of different mutations [177]. As such, *U. maydis* bring together genetic accessibility, easy and rapid cultivation methods similar to bacteriological techniques, and the power to use sophisticated molecular tools.

Apart from the traditional advantages, this filamentous fungus is a suited model to study the organization of MT by molecular motors [171, 174]. Several studies have reported discoveries in *U. maydis* related to organelle motility that later were confirmed in the mammalian system [171, 175, 176]. In fact, hyphal growth occurs at the tip, requiring transport of vesicles and organelles along the components of the cytoskeleton, basic long-distance transport, and motor-based microtubule organization mechanisms that seem to be conserved from fungi to mammals.

Furthermore, neurons and filamentous *U. maydis* are phenotypically similar. In both cases, unlike most cell types, MTs are not nucleated at a MTOC and form extensive arrays that serve as “tracks” for intracellular long-distance transport along the hyphal cell or axon [173]. Also, as previously mentioned, in neurons the anterograde and retrograde transports are mediated by kinesin and dynein, respectively, and specifically, the transport towards the axonal growth cone is mainly based on kinesin-1 and kinesin-3 motors [178]. Curiously, in the filamentous form of *U. maydis*, hyphal growth requires motor from the kinesin-1 and kinesin-3 family as well [173, 179, 180]. Another evidence of *U. maydis* and neurons conserved long-distance transport MTs-based machinery is the presence on both organisms of Rac-1, a small GTPase involved in neuronal polarity and polarized hyphal growth regulated by neuronal Cdk5 and Cdk5-like kinase in neurons and *U. maydis*, respectively [181-183]. Finally, analysis based on RNA-binding proteins in *U. maydis* indicates that MTs are required in long-distance transport of RNA in the filamentous form of the fungus, suggesting one more time that due to the phenotypical resemblance between neurons and hyphae *U. maydis*, similar transport mechanisms are used [173].

In this respect, *U. maydis* combined technical amenability with relevant neuron-like biology, owning important features for an HTS model for the screening of MT-stabilizing compounds.

Chapter II

Aim of the dissertation

Neurodegenerative disorders are a group of different diseases, e.g. AD, PD, ALS and many others, all characterized by a progressive loss of specific neuronal populations leading to a decline in brain function. These pathologies affect millions worldwide, and the numbers will continue to increase, given the ageing of the general global population, a risk factor for the development of neurodegenerative disorders. Furthermore, neurodegenerative disorders are one of the biggest challenging diseases to effective drug discovery. The traditional drug development process can take 10-15 years, involving huge costs [184]. Starting with thousands of molecules in every drug screen pipeline, hundreds can make the preclinical phase, however, approximately 10 reach the human clinical trials, and less than 1% is approved [184]. The statistics are even worse for neurodegenerative disorders, as the development of new drugs have very low success rate. Thus, it is fundamental to develop new tools for the discovery of a disease-modifying drug. Currently, the therapeutics available only manage the physical and mental symptoms.

The different neurodegenerative disorders can vary in neural cell type and the region in the brain. However, they seem to have in common dysfunctional axonal transport, a process supported by MTs. Indeed, different studies show that dysregulation of MT has been observed in wide range of neurodegenerative disorders [105]. Consequently, these observations led to an interest in assessing microtubule-stabilizing agents as a treatment, creating a demand for more capable research tools in this field. Alternative models have been shown to reduce drug discovery time through the use of HTS.

The fungus *U. maydis* combine features for HTS with relevant neuronal-like biology, since neurons and the hyphal form of *U. maydis* are phenotypically similar and make use of the same mechanisms of MT transporters along the cell. Thus, *U. maydis* owns important features for an HTS model for the screening of drug candidates to MT-stabilizing compounds.

Previously it was initialized in the group the establishment of a *U. maydis* HTS model towards drug repurposing in the axonal pathologies, specifically the screen of potential MT-stabilizing drugs. For that, *U. maydis* was used to screen the Prestwick chemical library, a library of FDA-approved drugs containing 1280 compounds.

Consequently, the major aims of this project were:

1. Analysis of *Ustilago maydis* high-throughput screening
2. Establishment of tools to validate positive candidates in neuronal cells
3. Development of a BBB-on-a-chip

To present the work developed and the data collected this document was divided in six main chapters.

Chapter I introduces this work, consisting of a literature review. It focuses on different topics, being divided in four main groups: the neurodegenerative disorders paradigm, the microtubule role, the importance of drug repurposing towards neurodegenerative treatment, and finally, the features of *Ustilago maydis* and how can it be an HTS model in neurodegenerative disorders.

Chapter II reflects the aim of the work, highlighting the major objectives and presents the structure of the dissertation.

Chapter III, IV, and V focus on presenting the results and discussion regarding the main objectives of the dissertation, confirmation of *U. maydis* HTS, the establishment of tools to validate positive candidates in neuronal cells, and BBB-on-a-chip, respectively.

Finally, Chapter VI summarizes the main conclusions and proposes future considerations. An appendix is also provided with *U. maydis* HTS further analysis, image analysis tools for ND7/23 cells and primary cortical neurons models, 3D printed BBB-on-a-chip templates and contact angle measurements for surface tailoring evaluation.

Chapter III

Ustilago maydis high-throughput screening

Introduction

In the hyphal form of *U. maydis*, microtubules have an essential role in polar growth, nuclear migration, and are fundamental for MT-based transport of vesicles and organelles along the cell. Interestingly, the fungus *U. maydis* and human mammalian cells show a level of conservation of the MT machinery between the species [180, 185].

Table 8. Microtubule-associated proteins (MAPs) involved in MT stabilization and polymerisation and their distribution among *U. maydis*, *Homo sapiens* and *Saccharomyces cerevisiae*.

Microtubule-associated protein (MAP)	<i>Ustilago maydis</i>	<i>Homo sapiens</i>	<i>Saccharomyces cerevisiae</i>
Bim1	Present ●	Present ●	Present ●
Kip2	Present ●	Present ●	Present ●
Pac2	Present ●	Present ●	Present ●
Peg1	Present ●	Present ●	Present ●
NudM	Present ●	Present ●	Present ●
AURKA	Present ●	Present ●	Present ●
CAMSAP2	Not present ●	Present ●	Not present ●
CAMSAP3	Not present ●	Present ●	Not present ●
CAPN6	Present ●	Present ●	Not present ●
CKAP2	Not present ●	Present ●	Not present ●
CLASP1	Present ●	Present ●	Present ●
CLASP2	Not present ●	Present ●	Not present ●
CLIP1	Present ●	Present ●	Present ●
Bim1	Present ●	Present ●	Present ●
Bin2	Present ●	Present ●	Present ●
Bin3	Present ●	Present ●	Present ●
MAPT	Not present ●	Present ●	Not present ●

The expression of multiple tubulin isotypes is observed both in human cells and in *U. maydis*. Accordingly to the bioinformatic evaluation of MIPS database by Shiel, A. [185] *U. maydis* has one α -tubulin gene, two genes encoding different β -tubulins, and one gene for γ -tubulin. Also, some MAPs, an important piece in the regulation of MT dynamics, involved in MT stabilization and polymerisation are present both in the *U. maydis* and in *H. sapiens* (Table 8). However, one should always take into consideration the distinct complexity between both species, thus the need for more tubulin isotypes and MAPs in the MT machinery in human cells.

Besides *U. maydis*, other species of filamentous fungi emerged as model organisms for studying MT-based transport for cellular tracking, such as *Aspergillus nidulans*. Similar to *U. maydis*, the other filamentous fungi have in their long hyphal compartments regions in which polarized MTs are oriented in uniform or anti-parallel networks (Fig. 6) [186]. So, why *U. maydis* would be different from the other filamentous fungi in mimicking mammalian neurons?

Besides the similar MT-transport mechanism, as previously mentioned in chapter II, section 4, the filamentous form of *U. maydis* is phenotypically similar to mammalian neurons (Fig. 6), owning a MTs unipolar array and a structure very similar to mammalian neurons (namely the axons), contrary to *Aspergillus nidulans* [186].

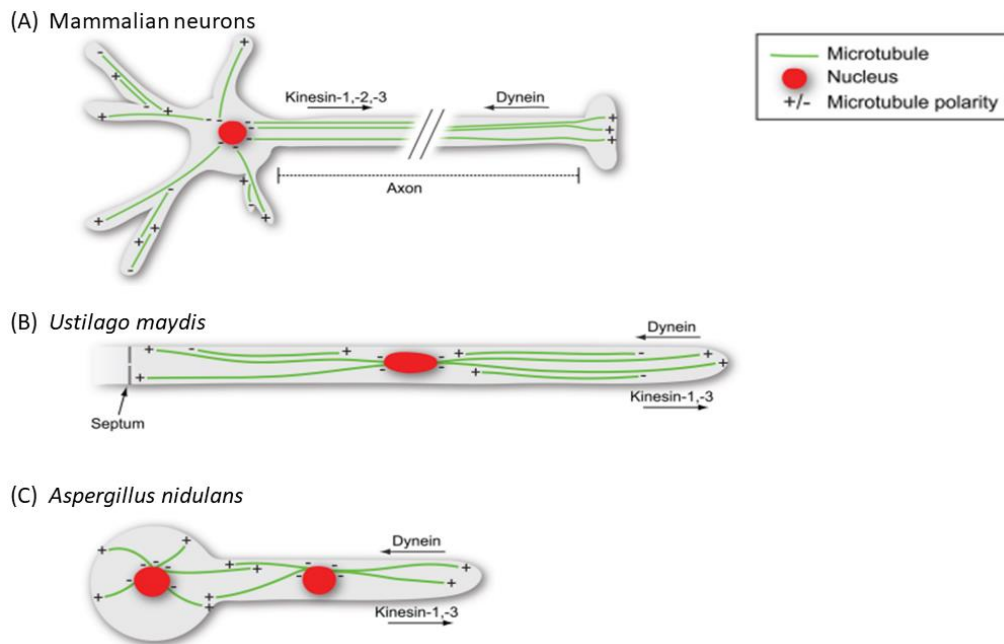


Figure 6. Microtubule polar growth and organization in mammalian neurons and filamentous fungal hyphae. A) Mammalian neurons MTs polar growth (unipolar in axons and antiparallel in dendrites); B) *Ustilago maydis* filamentous form with MTs polar growth (uniformly oriented); C) *Aspergillus nidulans* MTs polar growth (mixed polarity). Based on [186].

Therefore, *U. maydis* could be a simple, accessible, and adequate whole-organism high-throughput platform for drug screening. Namely, libraries of regulatory agencies-approved drugs, designated as “drug repurposing”. This novel alternative model can fast-track the transition from bench to bedside.

Materials and Methods

1.1. Fungus strains and growth conditions

The *U. maydis* strain used in this study was TUBB1 (AB33GTub1GSKL). The strain was cultured in complete medium (CM) (Table 9) supplemented with 1% (w/v) glucose at 28°C and 180 rpm. To induce hyphal growth in all strains the medium was shifted to nitrate minimal (NM) medium (Table 9).

Table 9. Solutions used in medium growth of *Ustilago maydis*.

Solution	Reagents	Ratio
Vitamin Solution	Thiamine hydrogen chloride	0.1% (w/v)
	Riboflavin	0.05% (w/v)
	Pyridoxine hydrochloride	0.05% (w/v)
	D-pantothenic acid hemicalcium salt	0.2% (w/v)
	4-aminobenzoic acid	0.2% (w/v)
	Nicotinic acid	0.2% (w/v)
	Choline chloride	0.2% (w/v)
	Myo-inositol	0.2% (w/v)
Deionised H_2O		
Trace Elements	Boric acid	0.06% (w/v)
	Ferric acid. $6H_2O$	0.01% (w/v)
	Sodium molybdate. $2H_2O$	0.4% (w/v)
	Zinc chloride	0.04% (w/v)
	Manganese (II) chloride. $4H_2O$	0.14% (w/v)
	Copper (II) sulphate. $5H_2O$	0.04% (w/v)
Deionised H_2O		
Salt Solution	Monopotassium phosphate	16% (w/v)
	Trace elements	8.0% (w/v)
	Calcium chloride. $2H_2O$	1.32% (w/v)
	Magnesium sulphate. $7H_2O$	4.08% (w/v)
	Potassium chloride	8.0% (w/v)
	Sodium sulphate	4.0% (w/v)
Deionised H_2O		
Complete Medium (CM)	Casamino acids	0.25% (w/v)
	Yeast extract	0.1% (w/v)
	Vitamin Solution	1.0% (w/v)
	Salt Solution	6.25% (w/v)
	DNA (from herring sperm)	0.05% (w/v)
	Ammonium nitrate	0.15% (w/v)
Deionised H_2O , pH 7.0		
Nitrate minimal (NM) Medium	Potassium nitrate	0.3% (w/v)
	Salt Solution	6.25% (w/v)
Deionised H_2O , pH 7.0		

1.2. HTS of Prestwick Chemical Library

The first step is to induce hyphal formation. For that, a small amount of *U. maydis* is collected from the agar plate to a 100 mL baffled flask (allows the improvement of aeration) with CM supplemented with 1% glucose overnight at 28 °C, 180 rpm. Then, the solution is centrifuged at 3000 rpm for 10 minutes, the supernatant is removed and resuspended in NM medium supplemented with 1% glucose. Once again, the cells are centrifuged at 3000 rpm for 10 minutes, the supernatant is thoroughly removed, and the pellet is resuspended again in NM medium supplemented with 1% glucose.

Afterwards, in a 50 mL conical tube the appropriate volume of the cells was added to the culture until a OD_{600nm} 0,350 was reached. The culture was placed in a 500mL baffled flask for 2h at 180 rpm, 28°C. After 2h the OD_{600nm} was measured (around 0.500), and 100 μ L of culture was dispensed per well into a black 96-well plate with clear bottom. Through the use of an automated liquid handling workstation (JANUS, PerkinElmer, Biosciences Screening Platform, i3s, Porto) 1 μ M of each compound of Prestwick Chemical Library (1280 FDA- & EMA-approved drugs) were added. The samples were incubated at 28°C in a plate orbital agitator at 450 nm. After, the absorbance (OD_{600nm}) and fluorescence (488nm – 590nm) were measured in a multi-mode plate reader (BioTeck Synergy). From the analysis of all compounds 50 were picked for dose-response analysis. The procedure is similar to the one described but the drugs were added in 4 different concentrations (4 μ M; 2 μ M; 1 μ M; 0.5 μ M). This assay was previously performed in the lab.

1.3. Bioinformatic analysis

Bioinformatic/ statistical analysis was performed using GraphPad Prism (version 8, www.graphpad.com), Venn diagrams were created with a bioinformatic tool (www.bioinformatics.psb.ugent.be/webtools/Venn/) and the bioinformatic prediction tool admetSAR was used for assesement of chemical properties of compounds.

Results and Discussion

2.1. Bioinformatic analysis of *U. maydis* HTS for microtubule-stabilizing compounds

As a proof-of-concept for *U. maydis* as a proper model for the development of microtubule-stabilizing drugs, it was previously performed in the group an HTS by testing the Prestwick Chemical Library in the hyphal form of the fungus *U. maydis*. This set of 1280 bioactive compounds included drugs for different therapeutic classes (Fig. 7), allowing the analysis of various compounds and their possible role for drug repurposing as microtubule-stabilizing drugs. Fig. 6 shows the distribution of the bioactive compounds of Prestwick Chemical Library accordingly with their therapeutic class, with 15 different classes.

50 compounds arose from HTS as potential hits. After, dose-response analysis was performed, 22 hits showed a microtubule-stabilizing effect in *U. maydis*, (Fig. 8, an explanation regarding these results is given in Appendix A).

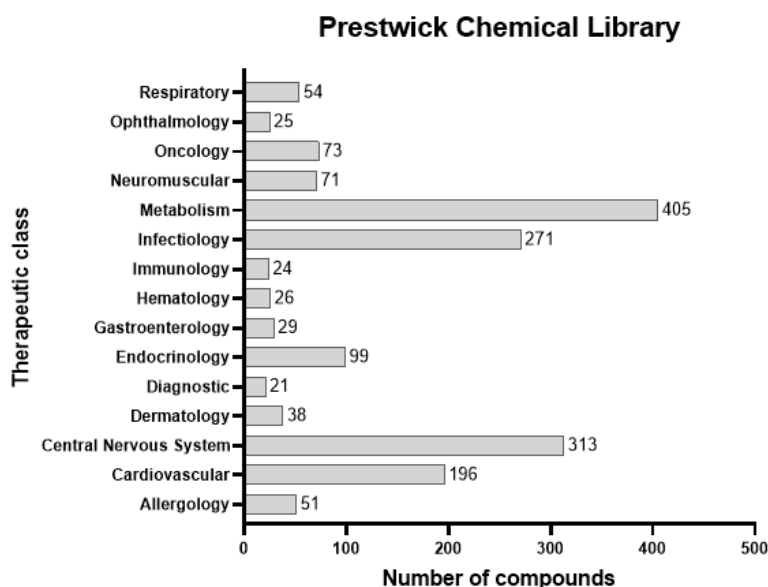


Figure 7. Distribution of the 1280 bioactive compounds of Prestwick Chemical Library *per* therapeutic class, to be noted that one compound can be included in more than one therapeutic class.

Ustilago maydis HTS with Prestwick Chemical Library					
⊕	Positive Candidates			⊖	Negative Candidates
	• Fulvestrant • Atracurium besylate • Benzonatate • Mexiletine hydrochloride • Flavoxate hydrochloride • Disulfiram • Troleandomycin • Danazol • Dronedarone hydrochloride	• Hexetidine • Mitoxantrone dihydrochloride • Dequalinium dichloride • Tobramycin • Flubendazole • Bosentan • Pranlukast • Monensin sodium salt • Carbenoxolone disodium salt	• Alexidine dihydrochloride • Novobiocin sodium salt • Ethaverine hydrochloride • Aprepitant		• Niclosamide • Chloroxine • 6-Furfurylaminopurine • Benzoxiquine • Pyrvinium pamoate

Figure 8. List of Positive (22) and Depolymerizing/negative (5) hits retrieved from the *Ustilago maydis* HTS with Prestwick chemical library.

Fig. 9 shows the distribution of the positive hits accordingly to their therapeutic class. Also, from the screening 5 candidates with depolymerising capacities arose. Although not in the scope of this work, these compounds might be of interest for future studies. Thus, from the 15 initial therapeutic classes evaluated only 8 are included in the positive hits: respiratory, oncology, neuromuscular, metabolism, infectiology, endocrinology, cardiovascular, and central nervous system. Notably, from the 313 compounds within the therapeutic class on CNS, only 1 showed a pronounced microtubule-stabilization effect on *U. maydis* model. To be noted that the positive candidates were selected according to the strongest phenotype. So, some candidates known to stabilise the MT (e.g. Taxol) did not arise as positive hits because other drugs produce a stronger effect. However, one should also consider the hypothesis that this can in part be a reflection of a shortage effect of CNS drugs in neurodegenerative disorders where MT destabilization is an important abnormality. On the other hand, metabolism and infectiology are the therapeutic classes with more positive hits, each of them represented by 8 drugs.

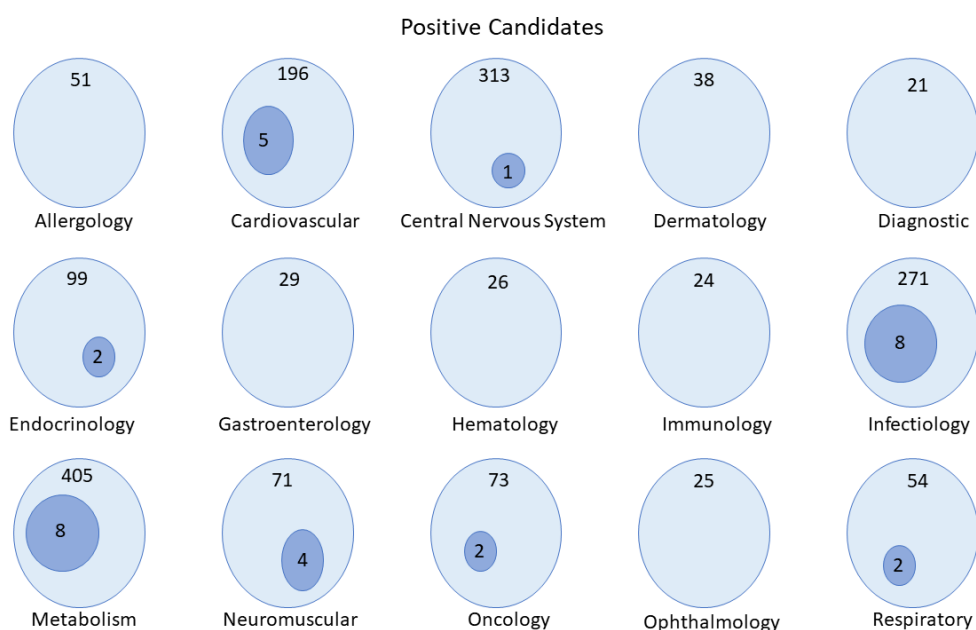


Figure 9. Distribution inside the Prestwick Chemical Library therapeutic classes of the positive candidates (represented darker blue circles) given by the HTS in *U. maydis*. Considering that one compound can be included in more than one therapeutic class.

A deeper look at the 22 positive candidates is represented in Fig. 10 and Table A1 (Appendix A), where one can see the distribution *per* therapeutic effect and specific properties of each candidate, respectively. For a more detailed analysis of the distribution of positive hits *per* therapeutic effect, it was considered all the effects described to each specific drug, since sometimes therapeutic effect – therapeutic classes do not match completely. This is a sign of drugs' capacities to possess different therapeutic effects, an important feature for drug repurposing.

The 22 positive candidates represent 20 different therapeutic effects, where antineoplastic and antibacterial stand out as the ones with the highest number of bioactive compounds showing microtubule-stabilizing effect (Fig.10 (A)). There are 12 therapeutic effects (antabuse, anti-estrogen, antihelmintic, gap junction, antispastic, antiemetic, vasodilator, antiasthmatic, proton

ionophores, antiprotozoal and anti-ulcer) that are only represented by one compound. These results can, perhaps, translate an unspecific relation between these therapeutic effects and the microtubule-stabilization capacity demonstrated. Since, the compounds that correspond to these therapeutic effects are also associated with other therapeutic effects (e.g. antibacterial or antineoplastic) that are more common between the positive hits (the relation between the different groups of therapeutic effects is demonstrated in Venn diagrams present in Fig. 10 (B) and Appendix A). Nevertheless, the fact that HTS showed a higher number of compounds with antibacterial and antineoplastic effects as positive hits for microtubule-stabilizing phenotype it's a promising indicator of a successful screen with *U. maydis*.

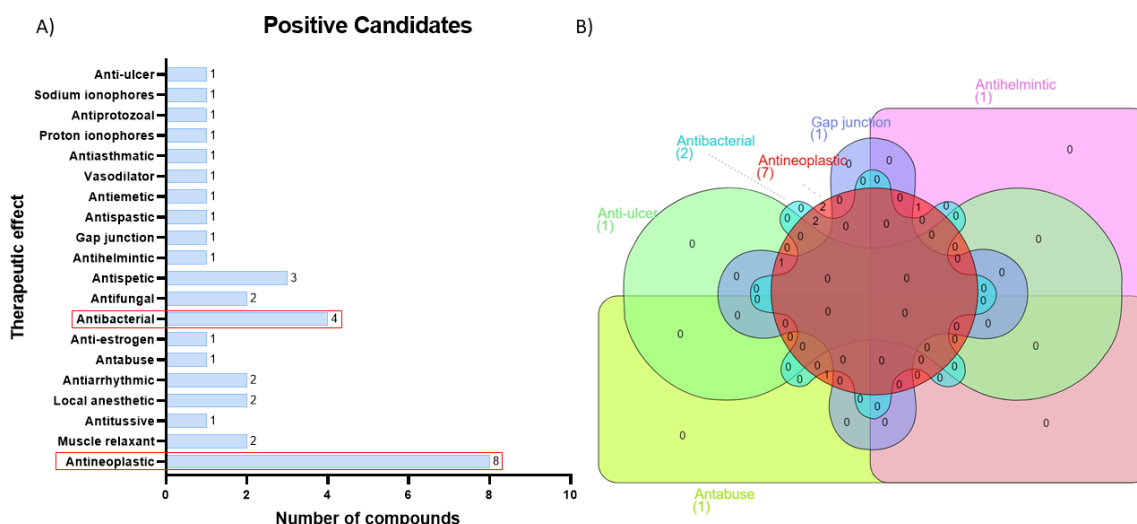


Figure 10. Distribution of positive candidates *per* therapeutic effect and their interconnection. A) Distribution of positive candidates *per* therapeutic effect. Red rectangles highlight the therapeutic classes with a higher number of positive candidates. It must be taken into consideration that each compound can be included in more than one therapeutic effect group; B) Venn diagram shows the relation and interconnection between compounds with antineoplastic and, at the same time, other therapeutic effects.

Given the uncontrolled growth characteristic of cancer cells and the crucial role of MTs in the mechanism of cell division MTs have been considered a target in cancer therapy. Microtubule-stabilizing agents are mostly used in cancer treatments, such as Paclitaxel that exerts a broad-spectrum antineoplastic activity, being one of the most used chemotherapeutic drugs. Curiously, was also the first used microtubule-stabilizing drug as a proof-of-concept in the treatment of neurodegenerative disorders. Furthermore, cancer and neurodegenerative diseases share abnormalities such as mitochondrial dysfunction, oxidative stress, and the development of misfolded proteins, which turn antineoplastic drugs into strong candidates in the repurposing process for neurodegenerative treatment. Therefore, it was expected that some bioactive compounds with indications for antineoplastic effects also showed the capacity for microtubule-stabilization, which was translated in the HTS results with antineoplastic agents representing the biggest therapeutic effect within the positive hits.

Antibacterial agents have also previously been studied for their potential suitability in the treatment of neurodegenerative disorders. There has been experimental evidence that antibiotic drugs exert neuroprotective effects. Macrolide antibiotics, in particular, have been used in studies to treat AD and the results showed a decrease of amyloid- β in the brain [136,

139]. The fact that antibacterial and antineoplastic were the therapeutic effects with more incidence of positive candidates aligns with the repurposing studies for neurodegenerative treatments ongoing [136, 137]. This data is an indicator that *U. maydis* can be an appropriate model for HTS of drug libraries for MT modulation.

2. Selection of most promising positive candidates: pursuing to mammalian models evaluation

Regarding the bioactive compounds that showed microtubule-stabilizing properties in *U. maydis* model 5 compounds were selected (Fig. 11) to be evaluated in neuronal cells, allowing the analysis of these in microtubule-stabilizing effect in mammalian models. Aprepitant, Atracurium besylate, Danazol, Disulfiram, and Pranlukast represent the diversity of therapeutic effects presented in the universe of all the 22 positive hits.

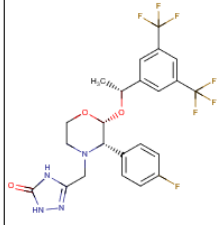
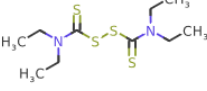
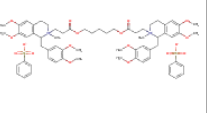
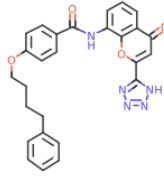
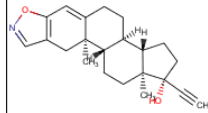
Molecular Structure	Drug				
	Aprepitant	Disulfiram	Atracurium besylate	Pranlukast	Danazol
					

Figure 11. Schematic representation of the molecular structure of each selected positive candidate: Aprepitant, Disulfiram, Atracurium besylate, Pranlukast, and Danazol.

A thorough bioinformatic analysis of each selected candidate on: physicochemical properties; and mode of administration, storage conditions and solubility, biological half-time, and side effects; are described in table 10 (more information regarding drug targets and relevant studies reported in the literature are present in Appendix A, table A1).

Until now, to the best of our knowledge, no studies were reported on Aprepitant and Danazol having relevant therapeutic targets for the neurodegenerative or within neurological field. However, Pranlukast was reported by Li-sheng et al. [187] as capable of neuroprotective effect, ameliorating neuron degeneration. While Disulfiram was pointed as a novel enhancer of ADAM10 gene expression (a gene involved in Alzheimer's disease) by Reinhartdt et al. [188], and Atracurium besylate promotes astroglia differentiation [189].

Nevertheless, none of the selected compounds has ever been used in clinical trials for neurodegenerative or neurological disorders (clinical trials for each candidate are presented in Table 10). However, the data on BBB permeability is scarce. Although drug capacity to pass the BBB is not *per se* a limitative step for drug development in the field of neurodegenerative disorders, it is an important drug property. So, through admetSAR a bioinformatic prediction tool for assessment of chemical ADMET properties, an expected BBB permeability for each compound is displayed in Table 10. Interestingly, all the 5 selected candidates, theoretically, have the capacity for crossing the BBB.

Table 10. Physicochemical properties and relevant features of selected positive candidates: Aprepitant, Disulfiram, Atracurium besylate, Pranlukast, and Danazol.

Selected Positive Candidates									
Drug	Formula	Molecular Weight (g/mol)	Solubility and storage	Mode of administration and administered dose	Side effects	Biological half-time	Clinical trials	Expected BBB permeability	Reference
Aprepitant	$C_{23}H_{21}F_7N_4O_3$	524.44	DMSO and ethanol Stored at Room Temperature	Oral 40,80 and 125 mg	Anorexia	9-13 hours	Chemotherapy-induced nausea and vomiting Parkinsonian type disorders	Yes	[190-194]
					Constipation				
					Dyspepsia				
					Fatigue				
					Headache				
					Hepatic enzyme levels elevated				
Disulfiram	$C_{10}H_{20}N_2S_4$	296.54	DMSO Storage at 15-30°C	Oral 800 mg (1st day) and 200 mg to 100 mg rest of the treatment	Hiccup	7.3 hours	Variety of conditions	Yes	[195-198]
					Antabuse				
					Confusion				
					Diarrhea				
					Epigastric misery				
					Fatigue				
					Headache				
					Memory lost				
					Nausea				
					Optic nerve damage				
Atracurium besylate	$C_{65}H_{82}N_2O_{18}S_2$	1243.51	DMSO, ethanol and H_2O Storage at -20°C	Intravenous 10 mg/mL	Somnolence	0.33 hours	Variety of conditions, namely for anaesthetic (blocking agent)	Yes	[199-201]
					Bradycardia				
					Bronchospasm				
					Dyspnea				
					Hypertension				
					Hypersensitivity reaction				
					Hypotension				
					Tachycardia				
Pranlukast	$C_{27}H_{23}N_5O_4$	481.52	DMSO Stored at Room Temperature	Oral 50 or 225 mg	Vasodilation	3-9 hours	Only for allergic rhinitis	Inhibits/Yes	[202-205]
					Arthralgia				
					Fever				
					Headache				
					Myalgia				
Danazol	$C_{22}H_{27}NO_2$	337.47	DMSO	Oral 50,100 and 200 mg	Upper respiratory tract infection	4.7 hours	Variety of conditions, namely endometriosis	Yes	[206-209]
					Acne				
					Anxiety				
					Headache				
					Hepatic enzyme levels elevated				
					Menstrual disturbances				
					Pruritus				
					Weight gain				

Conclusion

U. maydis filamentous form phenotypically similarity with human neurons and the level of shared MT-transport mechanisms between both organisms makes it a promise whole-organism model for HTS of microtubule-stabilizing compounds.

The HTS of Prestwick chemical library, a group of regulatory agencies approved drugs, hinges the valuable characteristic of *U. maydis* model and drug repurposing approach. This combinatorial approach in pursuit of microtubule-stability agents is an attempt to fast-track the transition from bench to bedside.

The first task developed in this project was the analysis of the HTS in *U. maydis*, previously performed in the group. From the study, it was retrieved 22 positive (MTs stabilizers) and 5 negative (destabilisers) hits from the 1280 bioactive compounds included in the library. For deeper bioinformatic analysis of these results, their distribution *per* therapeutic class and *per* therapeutic effect were quantified. Indeed, from the 15 therapeutic classes evaluated only 8 are represented between the positive candidates with an emphasis on metabolism and infectiology.

Important to note that from the 313 compounds representing CNS therapeutic class included in the Prestwick Chemical Library, only one displays the capacity for microtubule-stability in the *U. maydis* model. This result can be an indication that indeed the CNS drugs approved are either not targeting the MTs or not having a relevant stabilizing effect on MT, which can explain the lack of effective therapies on CNS disorders.

Regarding therapeutic effects, it should be emphasized antineoplastic and antibacterial as the most represented between the positive hits. Accordingly to the literature, it can be a promising indicator of a successful *U. maydis* HTS since both antineoplastic and antibacterial have studies reporting their MT modulatory capacity.

From the 22 positive candidates, 5 compounds were selected: Aprepitant, Atracurium besylate, Danazol, and Disulfiram. The compounds would be further validated in the mammalian system within the established tools (Chapter IV) for accessing MT modulation.

Chapter IV

Development of tools to
validate positive
candidates in neuronal
cells

Introduction

To confirm the potential of *U. maydis* as a model for HTS of MT-stabilizing agents the hits from the initial large-scale screen need to be validated in mammalian models.

Although rodent primary cells and cell lines do not fully represent the complexity and features of human cells, *in vitro* cell culture systems still constitute the traditional approaches for drug development. These models demonstrate some predictive power and are valuable tools for the validation of hits identified in HTS [138, 210, 211]. Indeed, in neurodevelopment and neurobiology, neuronal cell cultures have been exploited as a powerful model to answer relevant questions, such as signalling pathways and drug evaluation [210].

Given the characteristics of cell lines, such as the easy scale-up, reproducibility, simple manipulation, reduce animal use, and cost-effectiveness many studies employ these cells as a model for drug evaluation. Therefore, here we established a tool for MT-stabilizing compounds validation using a dorsal root ganglia (DRG)-derived immortalized sensory neuronal cell line, the ND7/23 cell line. This cell line is similar to primary neuronal culture in terms of phenotype and MT dynamics, allowing to have neuronal model but with the advantages of reducing animal use, increasing reproducibility and offering greater suitability for throughput assays due to ease of maintenance in culture and high rate of growth [212]. This cell line when cultured in an undifferentiated stage do not present any neurites. However, after growth factor stimulation ND7/23 cells acquired a differentiated phenotype. During this stage, cells can present long and unbranched neurites and be stained with neuronal specific markers such as β III tubulin.

Nevertheless, every model has advantages and disadvantages (Table 11), and cell lines immortalization was acquired at the cost of losing some important features, thus presenting alterations in the endogenous expression of proteins and failing to reproduce *in vivo* features. As an alternative numerous drug development studies employ primary cells, which more closely resemble the *in vivo* functions [163].

Table 11. Advantages and disadvantages of cell line and primary cell culture for drug screening [163].

Characteristic	Cell line	Primary cell culture
Scale up	Yes (theoretically unlimited)	Limited
Genetic stability	No	Yes
Batch-to-batch	Homogeneous	Heterogeneous
Cost	Low	High
Use for compound screening	Yes	Yes
Use in high-throughput screening	Yes	Difficulties
Human relevance	Moderate	Moderate
Electrophysiological properties	Due to expressed channels	Endogenous
Proliferation capacity	High	Generally low

Primary cortical neurons cultures represent a suitable *in vitro* model as neurons can spontaneously form neuronal networks with neurite outgrowth and synapse formation resembling *in vivo* morphofunctional features [213]. Therefore, besides implementation a tool for validation of MT-stabilizing drugs in ND7/23 cell line, here was implemented a model with mouse primary cortical neurons.

Although *in vitro* cell models' culture in flasks and well-plates are the traditional and standard used in drug development, it is recognized the limitation of altered patterns of molecules expression and modified morphology. Contrary to *in vivo* conditions, in the traditional cell cultures, all the different structures of neuronal cells are exposed to the same environment and do not allow compartmentalized studies. So, at end of the 20th century, the establishment of microfabrication techniques allow the development of microfluidic systems to overcome the limitations of the traditional cell culture models (Table 12) [211].

Table 12. Differences between microfluidic and traditional cell cultures [211].

Characteristic	Microfluidic cell cultures	Traditional cell culture
Cell culture material	Often PDMS	Polystyrene or glass
Number of cells	Fewer quantities	Bigger quantities
Consumption of reagents	Lower	Higher
Use in HTS	Yes	Difficulties in primary cells
Analytical biosensors	Possibility of incorporation	Not incorporated
Medium Flow/Shear stress	Yes	No

A microfluidic chip is a moulded material with a set of microchannels connected together with a certain pattern in which cells or tissues can be cultured in a compartmentalized manner [214]. These microfluidic devices were developed in order to reproduce a complex environment in a simple system, appropriate for pharmacological manipulation, physiological input, and image acquisition [215]. Especially in neuroscience, microfluidic technology was quickly adopted. In 2003, Anne M. Taylor published the first study where a two-compartment microfabricated neuronal culture device was developed with the accomplishment of physical segregation of neuronal soma from axons [215].

The microfluidic platforms allow the tailoring of the environment around the axons by separating these extensions from the cell body, as it happens in *in vivo*, providing a more complex and closer to *in vivo* model reducing to the minimum necessary resorting to animals [215, 216].

In the particular case of neuropharmacology, the microfluidic technology makes it possible to study the extension of axonal guidance, synapse formation and axonal formation, namely to evaluate MT stabilization as a response to different compounds.

Materials and Methods

1.1. Cell lines and culture conditions

ND7/23 cell line (mouse neuroblastoma (N18 tg 2) x rat dorsal root ganglion (DRG) neuron hybrid) was obtained from ECACC (United Kingdom).

ND7/23 cells were grown in Dulbecco's Modified Eagle Medium (1X) GlutaMAX™ high glucose (DMEM (1X) GlutaMAX™ high glucose, 31966-021, Gibco) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (F7524, Gibco) and 1% (v/v) Penicillin-Streptomycin (P/S) (L0022, BioWest). The cells were maintained in a humidified chamber in 5% CO₂ at 37°C.

The ND7/23 cells were detached at 80-90% confluency using 0.25% (w/v) trypsin/EDTA solution with 0.05% (w/v) glucose (Sigma-Aldrich) for 5 minutes at 37°C. Cell viability and density were assessed using trypan blue exclusion assay and cell concentration was adjusted for the one of interest. Afterwards, the cells were seeded on a pre-coated (overnight) 50 µg/mL Poly-D-lysine (PDL mol wt 70,000-150,000, P6407, Sigma-Aldrich) in 1M borate buffer, pH 8.5 24-well plate (µ-Plate 24 well uncoated sterile, ibidi) at a density of 4.000 cells/cm², 5.200 cells/cm², 6.300 cells/cm² and 8.000 cells/cm² and were grown in DMEM (1X) GlutaMAX™ high glucose supplemented with 10% (v/v) FBS, and 1% P/S (v/v) in 5% CO₂ at 37°C [212]. After 24 hours, to induce cell differentiation, the medium was changed to DMEM (1X) GlutaMAX™ high glucose supplemented with 1% (v/v) P/S, 0.5% (v/v) FBS, 1 mM Dibutyryl-cAMP (D0627-25MG, Sigma), and 100 ng/mL nerve growth factor (NGF, Mouse Submaxillary Glands, 480354-100UG, Calbiochem) [212, 217]. Cells were grown in 5% CO₂ at 37°C for 3 days. The cells were daily checked for follow-up of differentiated morphology and health conditions by using as inverted optical microscope.

1.2. Primary cell culture

1.2.1. Ethics statement

All experiments involving animals were performed in agreement with institutional ethical guidelines (IBMC/INEB/i3s), the EU directive (2010/63/EU) and Portuguese law (DL113/2013). All procedures were conducted by FELASA C graded researchers.

Animals had free access to food and water and all efforts were made to minimize the suffering and the number of animals used.

1.2.2. Primary mouse cortical neurons isolation and culture

Primary mouse cortical cell culture was kindly provided by M. Torrado, nBTT, i3s. First, cortical tissue was dissected from E15.5 mouse embryos. Then, the cortical tissue was dissociated using 1.5 mg/mL trypsin for 10-13 minutes at 37 °C, to a more efficient dissociation the conical tube of 15 mL was agitated occasionally. After dissociation the medium was removed, and the cortex was re-suspended in Hank's Balanced Salt Solution supplemented with 10% (v/v) FBS to inactivate the trypsination. Following, the medium was removed again, and Hank's Balanced Salt Solution was added to wash the cortex from the previously added FBS, hence preventing the growth of glia cells, such as astrocytes. This washing procedure with Hank's Balanced Salt Solution was repeated 3 times.

Afterwards, the cortex was re-suspended in neurobasal medium (12348-017, Gibco) and a mechanical dissociation was promoted, 10-20 times performing up and down with the pipette until the solution start to appear whitish, a signal of tissue dissociation. The solution was then filtered (cell strainer 70 μ m, 352350, BD Falcon) and the cells were counted with trypan blue exclusion assay. Finally, the cell solution was diluted with Neurobasal medium supplemented with 2% SM1, 0.05% L-Glutamic acid, 0.1% Gentamicin sulfate, and 0.25% glutamate to the adequate concentration needed for the conditions 39.500 cells/cm² and 26.300 cells/cm².

Finally, the cells were seeded in a pre-coated 50 μ g/mL PDL (overnight at 37°C) 24-well plate (μ -Plate 24 well uncoated sterile, ibidi).

1.3. Establishment of drug controls: DMSO, Nocodazole and Taxol

To analyse the microtubule-stabilizing effect first it was established controls with known effect drugs. For that, cells were incubated with 1 μ M nocodazole (M1404-10MG, Sigma-Aldrich), 100nM Taxol (027TXD01, Cytoskeleton, Inc.), and 1.0% DMSO. The pharmacological treatment was performed after 4 days in culture (3rd day post-differentiation) for ND7/23 cells, and at 7 days *in vitro* (DIV) for mouse primary cortical neurons.

1.4. Pharmacological treatment assay

Aprepitant (T1743, Tebu-Bio), Atracurium besylate (CSN16775, Tebu-Bio), Danazol (D8399-100MG, Sigma-Aldrich), Disulfiram (CSN10460, Tebu-Bio) and Pranlukast (CSN11761, Tebu-Bio) were assessed in the different models established for microtubule-stability agents' evaluation (ND7/23 cell line, primary cortical neurons model and microfluidic device) at a concentration of 100nM. The pharmacological treatment was performed after 4 days in culture (3rd day post-differentiation) for ND7/23 cells during 1h, 2h and 4h, and at 7 days *in vitro* (DIV) for mouse primary cortical neurons for 1h, 2h and 4h in the 2D model, and 48h in the microfluidic system.

1.5. Immunocytochemistry

For immunocytochemistry analysis cells were rinsed 3x with phosphate buffer saline (PBS) (Gibco) solution (and also after each step), and fixed in 4% (wv/v) MT- paraformaldehyde (MT-PFA) (Table 13) for 15 min at RT. Afterwards, the cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, at RT. The non-specific interactions were blocked with 5% bovine serum albumin (BSA) (BP1605-100, Fisher Scientific) in PBS for 45 min, at room temperature (RT).

Table 13. Reagents and respect concentrations of 4% MT-PFA.

Reagents	Concentration
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (H3375, Sigma)	25mM
PIPES (1,4-piperazinediethanesulfonic acid)	65mM
EGTA (egtazic acid)	10mM
MgCl ₂ (Magnesium chloride) (M8266, Sigma-Aldrich)	3mM
PFA (diluted in dH ₂ O)	8%
PBS	10X
NaOH	1mM

The cells were then co-stained with primary antibodies (1:500) diluted in PBS with 5% BSA (1:10) for 4h at RT preferably with agitation, followed by an incubation at 4°C overnight. After primary antibodies, the samples were incubated with secondary antibodies (1:1000) diluted in PBS with 5% BSA (1:10) for 45 min at RT, preferably with agitation. The combinations of the used primary and secondary antibodies are summarized in Table 14.

Table 14. Primary and secondary antibodies used in each model.

Model	Primary antibody	Secondary antibody
ND7/23 culture	Mouse Anti- α -acetylated tubulin (Sigma)	Anti-mouse Alexa-Fluor 594® (Invitrogen)
	Rabbit Anti- β III tubulin (Cell Signalling Technology)	Anti-rabbit Alexa-Fluor 488® (Invitrogen)
Primary cortical neurons culture	Mouse Anti- α -acetylated tubulin (Sigma)	Anti-mouse Alexa-Fluor 594® (Invitrogen)
	Rabbit Anti- β III tubulin (Cell Signalling Technology)	Anti-rabbit Alexa-Fluor 488® (Invitrogen)
Primary cortical neurons in Microfluidics	Rabbit Anti- β III tubulin (Cell Signalling Technology)	Anti-rabbit Alexa-Fluor 568® (Invitrogen)
	Mouse Anti- α -acetylated tubulin (Sigma)	Anti-mouse Alexa-Fluor 594® (Invitrogen)
	Alexa-Fluor 488® Flash Phalloidin (Invitrogen)	

Finally, the cells were incubated with Hoechst (ThermoFisher) diluted in PBS (1:5000) for 10 min at RT. When using ibidi 24-well plates (μ -Plate 24 well Uncoated sterile, ibidi) cells were left in PBS. On the other hand, when using 24-well plates (353047, Falcon) and 6-well plates (353046, Falcon) with coverslips, the samples were mounted using Mowioln-propyl-gallate and Fluoromount, respectively. Afterward, cells were observed under a confocal microscope (SP5, Leica, Germany) or IN Cell Analyzer 2000 (GE Healthcare).

1.6. Cellular metabolic evaluation – Intracellular [Ca^{2+}] measurements

To assess alterations in intracellular Ca^{2+} in response to control drugs and to positive candidates for microtubule-stabilizing agent it was performed a cellular metabolic evaluation using Fluo-4 AM assay [217].

Cells were seeded on a 50 μ g/mL PDL-coated black-walled 96-well plate with clear bottom (3603, Corning Incorporated) 4 days prior to the experiment (3 days prior differentiation) ND7/23 cells were seeded at a density of 8.000 cells/cm².

On the day of Ca^{2+} imaging the pharmacological treatment was performed prior to the Fluo-4 AM assay (F14201, Invitrogen by Thermo Fisher Scientific). After, the medium was removed and the cells were washed with 100 μ L/well Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (H2387-10, Sigma). 100 μ L/well of Fluo-4 AM working solution (approximately 3.5 ng/mL Fluo-4 AM in HBSS without Ca^{2+} and Mg^{2+}) were added to each well for 30 minutes at 37°C. To allow for complete de-esterification after the initial period of incubation the Fluo-4 AM working solution was removed, cells were washed again and incubated for another 30 minutes at 37°C with HBSS without Ca^{2+} and Mg^{2+} .

Finally, the results were evaluated in the multi-mode plate reader (Synergy Mx, BioTeck) and Ca^{2+} responses were measured with excitation at 485 nm and emission at 520 nm.

1.7. Microfluidic device

1.7.1. Microfluidic device preparation

To fabricate the microfluidic device polydimethylsiloxane (PDMS) base (SYLGARD™ 184 Silicone Elastomer Base, Cow) was mixed with a curing agent (SYLGARD™ 184 Silicone Elastomer Curing Agent, Cow) were vigorously mixed at a ratio of 10:1 (w/w%), and prior to being poured into the mould the mix was degassed in a desiccator connected to a vacuum pump. The crosslinking reaction between the PDMS base and the curing agent occurred at 70°C for 2h.

When polymerized the PDMS device was then peeled off from the mould and the inlet wells (6 mm diameter) of each chamber were created using a biopsy punch (6 mm, Kai medical)

Finally, the microfluidic devices were depurated with vinyl tape (3M Vinyl Tape 471 Yellow 50mm x 33m) followed by 15 minutes of UV or 70% ethanol sterilisation.

1.7.2. Fabrication of the microfluidic platform

Microfluidic platform was produced by pouring a mixed solution of PDMS with curing agent (10:1 ratio) into the mould.

The template was produced by our collaborators (Miguel Xavier, INL) to produce microfluidic devices that enable to isolate cell bodies from neurites. The template consists of 2 pairs of inlets, 2 channels, and a set of approximately 120 microgrooves. Each pair of inlets is linked by a channel on each side of the device. The channels are interconnected by the microgrooves, as illustrated in Fig. 12. The microgrooves total length is 450µm, with each being approximately 5µm x 10µm (height x width).

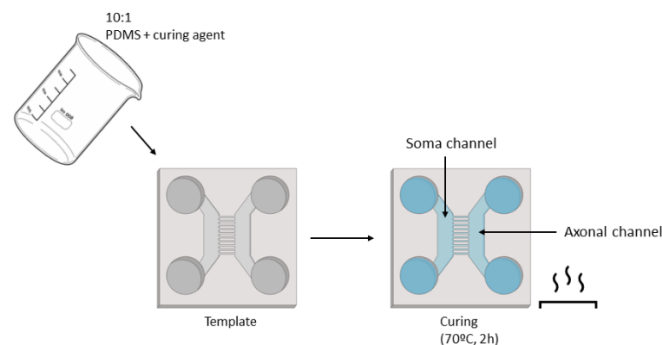


Figure 12. Microfluidic device produced by pouring a mix of PDMS with curing agent in a template (produced by collaborators), followed by a curing process.

1.7.3. Microfluidic device characterization

The morphology and dimensions of the microfluidic device were assessed by Scanning Electron Microscopy (SEM).

Samples were prepared, by sputtering, with a conductive coating of Au/Pd, a thin film of approximately 15 nm. The followed SEM analysis was performed using a High-resolution Scanning Electron Microscope with X-Ray Microanalysis (JEOL JSM 6301F/Oxford INCA Energy 350).

1.7.4. Cell seeding

First, the sterile microfluidics were bonded to coverslips pre-coated overnight at 37°C with 10 µg/mL Poly-D-Lysine (PDL mol wt 70,000-150,000, P6407, Sigma-Aldrich) in a 6-well plate (353046, Falcon).

Before the introduction of cells, supplemented neurobasal medium was added into both inlets, greater volume in one side to create a gradient flow.

The primary cortical neuron solution was centrifuge at 400g for 5 minutes and re-suspended in supplemented neurobasal medium in the volume needed to achieve the concentration of interest in each situation. Cells were introduced into one of the inlets of the device at a concentration of 500.000 cells/microfluidic, 800.000 cells/microfluidic, and 100.000 cells/microfluidic. Microfluidic device remained in a humidified incubator for up to 7 DIV prior to drug treatment assay and the media was replaced every 2-3 days.

1.8. IN Cell Analyzer 2000

For morphological evaluation of ND7/23 cells and primary cortical neurons drug treatment assays imaging was performed using IN Cell Analyzer 2000 (GE Healthcare), a fluorescence widefield microscope, and the obtained data was used for image-based quantifications.

In each experiment the wells imaging were acquired in a field with 49-equally spaced squares, in a 2D Deconvolution mode with binning 1x1 with the objective Nikon 20X/0.45. The acquired channels were DAPI (0.500 exposure), FITC (2.800 exposure), TexasRed (2.800 exposure), and Brightfield (0.100 exposure).

1.9. Image segmentation and analysis

For quantification of microtubule-stabilizing effect in ND7/23 cells, a method developed for oligodendrocytes by Bouçanova et al. [218] was adapted. Acquired IN Cell Analyzer 2000 images were pre-segmented using ilastik program (1.3.3 version, <https://www.ilastik.org/>), an open-source interactive learning and segmentation toolkit. Afterwards, further segmentation and analysis were performed using CellProfiler™ (3.1.5 version, <https://cellprofiler.org/>), a cell image analysis software. The final results were analysed with Microsoft Excel. A detailed explanation of the analysis process can be found in Appendix B.

For quantification of microtubule-stabilizing effect in primary cortical neurons a new method for quantification of the cellular brunching, using skeletonized images of the tubulin channel was created. Acquired IN Cell Analyzer 2000 images were segmented using ilastik program, followed by further segmentation and analysis using CellProfiler™. The final results were analysed with Microsoft Excel. A detailed explanation of the analysis process can be found in Appendix C.

1.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8, www.graphpad.com). For all datasets, tests for identification of outliers and normality were performed. To identify and remove likely outliers, the Robust Regression and Outlier Removal (ROUT) method with Q=10%

was used and Gaussian distributions were performed using the Shapiro-Wilk normality test ($\alpha=0.05$). Statistical differences between groups were calculated based on two-way ANOVA (influence of two factors). A p-value below 0.05 was considered statistically significant and data are shown as mean $\pm$ standard deviation (SD).

Results and Discussion

2.1. ND7/23 cell line: a tool to validate microtubule-stabilizing compound-candidates

2.1.1. ND7/23 differentiation

The ND7/23 cell line is a hybrid cell line from mouse neuroblastoma and DRG Neurons. When these cells are culture in the absence of differentiating factors, their phenotype is rounded, have no neurites, are scarce in neuronal specific tubulins (e.g., β -tubulin) and have high proliferation rates. Although maintaining their neuronal identity, these features difficult the study neuronal MTs and their dynamics. Nonetheless, literature reported that when differentiated the ND7/23 cells present long neurites and reduced cell division [212]. Here, differentiated ND7/23 cells were used to validate candidates for microtubule stabilization. Post-mitotic neuronal phenotype was achieved by culturing cells in the presence of NGF (a growth factor), and a high intracellular level of cAMP induced by dibutyryl-cAMP.

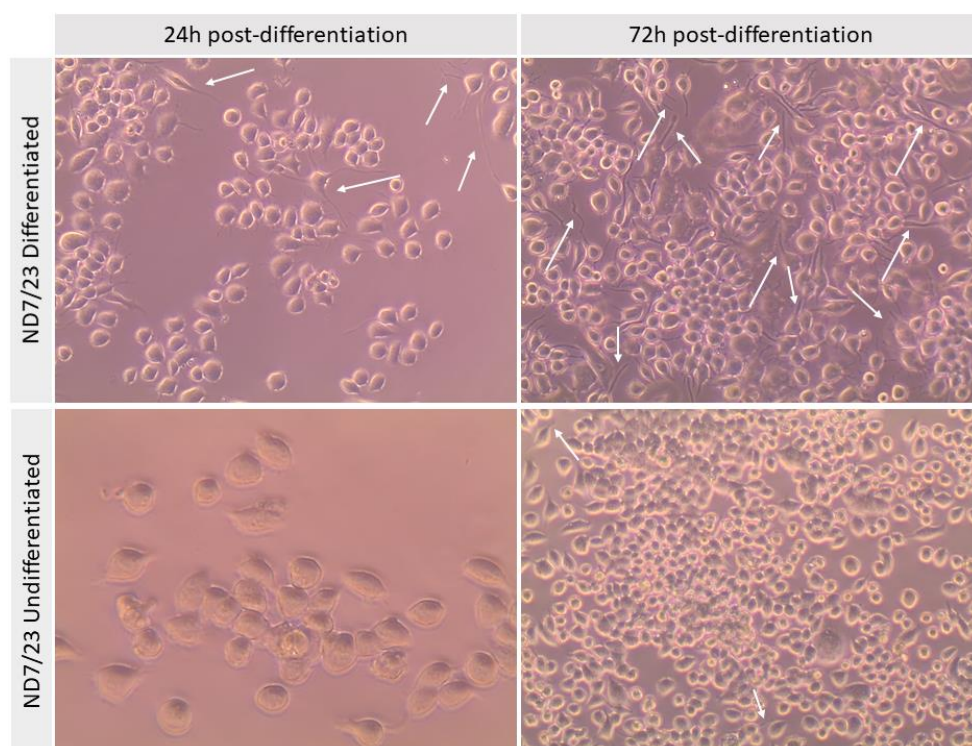


Figure 13. ND7/23 differentiation stages 24h (left side) and 48h (right side) post-differentiation compared with the same time points for undifferentiated ND7/23 cells. Images were obtained using an inverted optical microscope. Neurites are indicated by white arrows.

After 24h of differentiation, 20x optical microscopic examination showed that cells cultured in the presence of differentiated factors (Fig. 13, upper left side) present small and thin neurites, revealing the initiation of a neuronal morphology. On the opposite, undifferentiated ND7/23 cells maintained their rounded shape (Fig. 13, lower left side). At 48h post-differentiation, the neuronal morphology of the ND7/23 differentiated cells continued to evolve with the outgrowth of an increased number of long unbranched neurites (Fig. 13, upper right side). Although some undifferentiated cells presented short neurites, after 48h, the majority, maintained their rounded phenotype (Fig. 13).

The neuronal phenotype acquired by ND7/23 cells after induced differentiation is not only evident by the formation of neurites but is also accompanied by a visual reduction in cell division when compared to undifferentiated proliferation rates (Fig. 13). In *in vivo* conditions, neurons are cells with extremely reduced to no capacity to divide. Therefore, the high rate of proliferation presented by undifferentiated ND7/23 cells could mask the screening results for microtubule-stabilizing compounds since MTs are one of the major pieces in the cell division process. Accordingly, ND7/23 differentiated cells are an attractive alternative.

Given the extended phenotype presented by neuronal cell types *in vivo* and the role of MTs and their “dynamic instability” (Chapter I) when differentiated the ND7/23 cells can represent a better tool for neuronal validation of positive candidates than in their undifferentiated stage.

2.1.2. Optimization of ND7/23 cell seeding density

Neuronal phenotype is extensively influenced by cell density, as reported elsewhere [212]. Therefore, to assure that neuronal differentiation was properly achieved four different ND7/23 differentiated cell densities: 8.000, 6.300, 5.200, and 5.000 cells/cm² were tested. For the selection of the most appropriate cell density the ND7/23 cells were differentiated at each cell density, 24h after cell seeding, and at day 3 post-differentiation their morphology and phenotype were evaluated by staining β III- and α -acetylated tubulins (present in neuronal cells) and nucleus.

Fig. 14 (A) shows the stitch (IN Cell Developer Toolbox 1.9.3) of the 49 images acquired per well in the IN Cell Analyzer 2000. It is possible to observe that in higher cell densities (6.300 and 8.000 cells/cm²) the cells are evenly distributed allowing to mimic the cell-cell interactions similarly to *in vivo* situations. Contrarily, at 4.000 and 5.200 cells/cm² density it showed a very low confluency.

Comparing the two higher cell densities, the 8.000 cells/cm² seeding density showed a higher number of differentiated cells with visible neurites. Since differentiated cells have a low rate of proliferation the number of differentiated cells at the time point of drug treatment would generally be higher for the 8.000 cells/cm² seeding density.

Another aspect to be considered for the optimization of ND7/23 cell density, is the need to have individual differentiated cells to facilitate image analysis. Figure 14 (B) shows an example of images for single-cell analysis. Therefore, a commitment between the cell density, that should be physiologically relevant, and amenability to distinguish single cells in the images should be reached. Based on these observations, the 8.000 cells/cm² density was chosen to proceed in the development of the ND7/23 cell model.

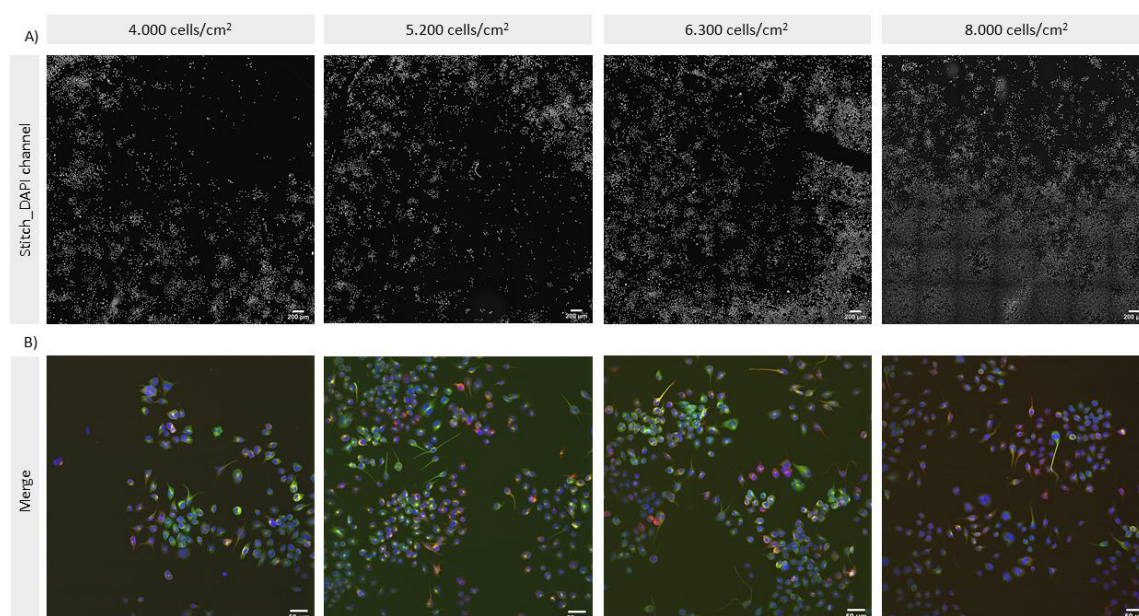


Figure 14. Representative images of ND7/23 differentiated cells in 4 cell seeding densities: 4.000, 5.200, 6.300, and 8.000 cells/well. A) Overlook obtained using stitch with DAPI channel of 49 images from IN Cell Analyzer 2000 for each cell seeding density. Scale bars: 200µm; B) Images of each cell seeding density representing proper locations for image analysis. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50µm.

2.1.3. Assessment of drug-controls for microtubule-stabilizing agents in ND7/23 differentiated cell model: DMSO, Nocodazole and Taxol

To use ND7/23 cell line as a suitable model to evaluate microtubule-stabilizing effects, the workflow present in Fig. 15 was established. Cells were seeded and differentiated, as previously reported and at day 3 the drug treatment was performed. Afterwards, images were acquired to investigate cell morphology and phenotype, followed by image analysis for quantification.

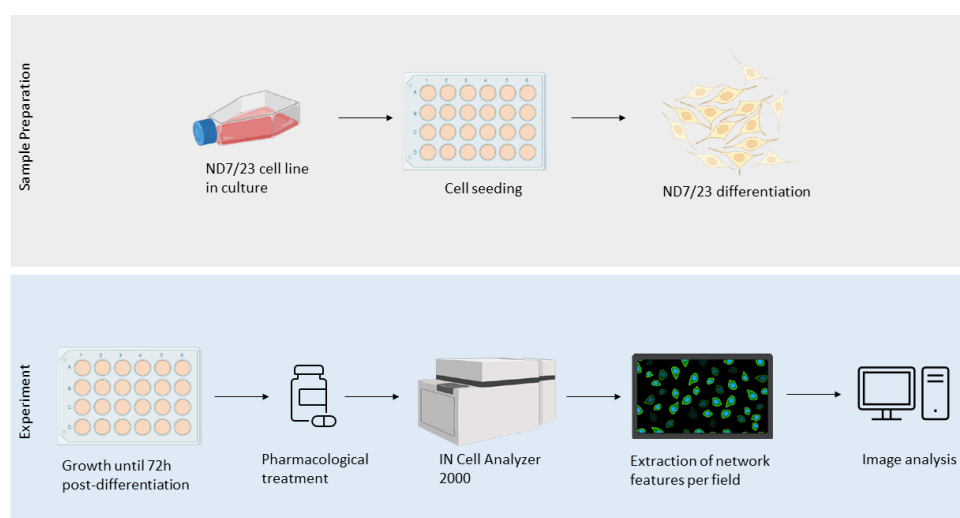


Figure 15. Schematic representation of the workflow followed for the establishment of ND7/23 cell line as a model for evaluation of microtubule-stabilization effect.

For studying microtubule-stabilizing effects, the drug nocodazole was used as a negative control since is described in the literature [219] as a microtubule destabilizer and as a positive control,

the extensively described microtubule-stabilizing drug Taxol [219] (also known as Paclitaxel, described in chapter I, section 2.5) was the selected compound. Since drug-controls and, also the positive candidates further evaluated were soluble in DMSO, this solvent was also used as a control.

At day 3 post-differentiation, the ND7/23 cells were treated either with 1.0% (v/v) DMSO alone or with 100nM Taxol or 1 μ M Nocodazole, for 4h. These concentrations were inferred from the literature for microtubule respective effects [213]. Cell morphology was evaluated by staining with β III tubulin (a neuronal specific tubulin) and α -acetylated tubulin (also present in neurons, but less prone to destabilization than β III tubulin). Fig. 16-18 shows representative images of ND7/23 model for DMSO, Nocodazole, and Taxol, respectively.

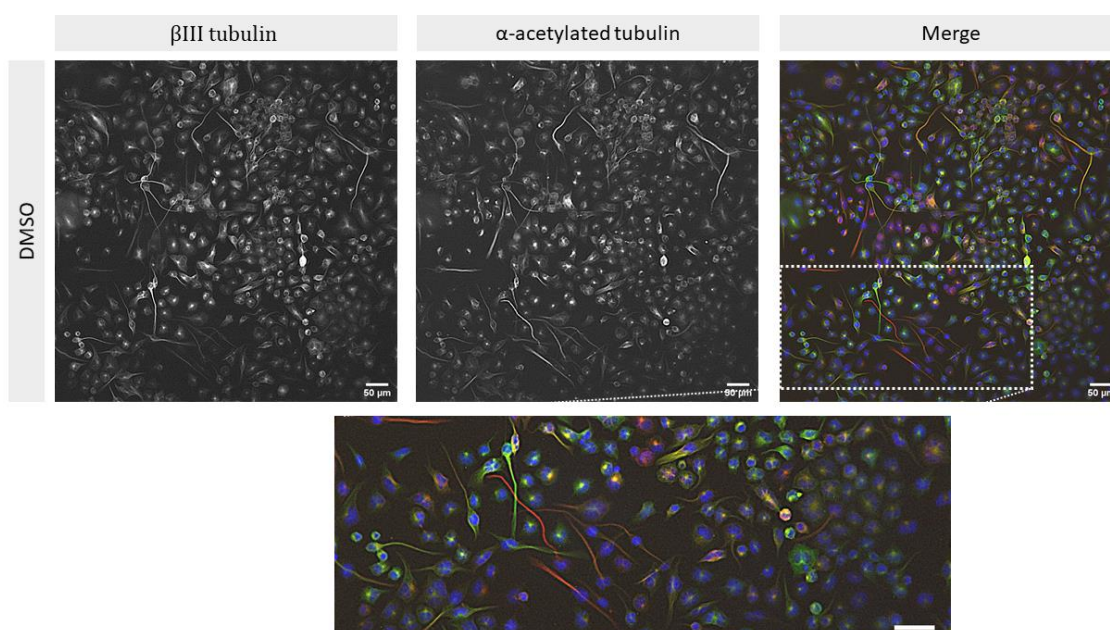


Figure 16. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at third day of induced differentiation and after 4h 1.0% DMSO treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

The use of 1.0% (v/v) DMSO is a commitment between drug solubility and cellular viability, since in high concentrations DMSO is toxic for cells. Nonetheless, as shown in Fig. 16 after 4h incubation the ND7/23 cells showed the neuron-like morphology developed after differentiation with thin extended neurites, not showing any signs of toxicity.

Looking at the negative control (Fig. 17), the antimitotic agent Nocodazole, after 4h of drug treatment, was able to induce microtubule-destabilization visible by a reduction in the density of α -acetylated and β III tubulins. It is also clear the shift of the initial differentiated morphology to a more rounded phenotype with alterations in the labelling of the tubulin cytoskeleton lost.

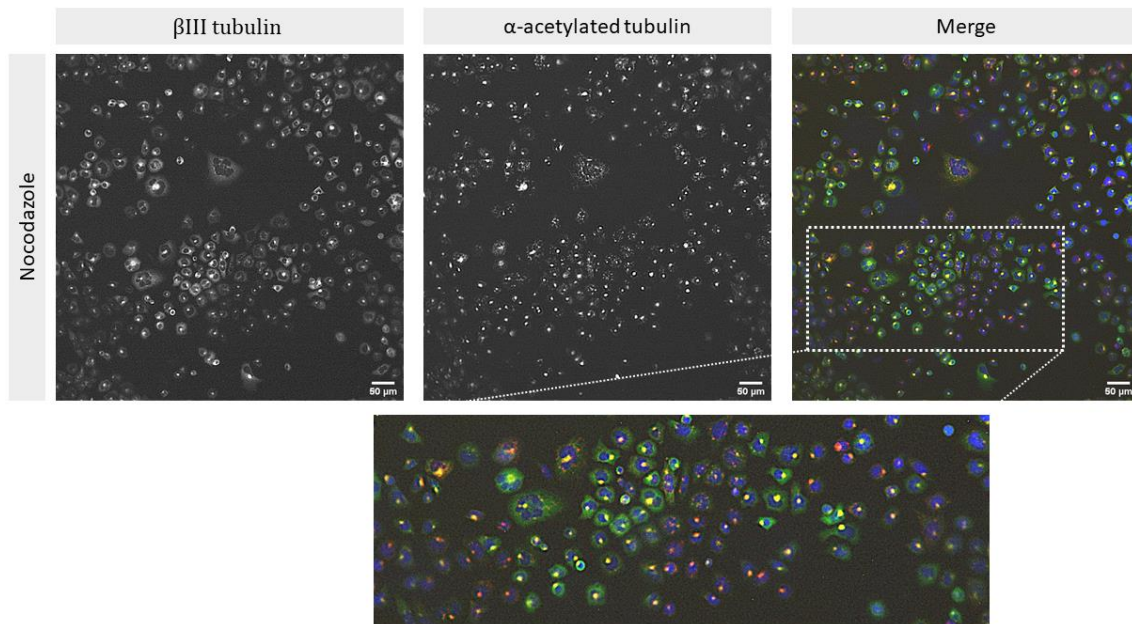


Figure 17. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at third day of induced differentiation and after 4h of 1 μ M Nocodazole treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

The effects of microtubule-stabilizer Taxol after 4h of drug treatment in ND7/23 cell line model can be seen by peaks of intensity in the staining of β III and α -acetylated tubulins (red arrow). The microtubule-stabilization of ND7/23 cells is also translated in a morphology alteration with cells passing from thin and extended neurites (Fig. 18) to a more constrained cytoskeleton with reduced length neurites but with a higher number of neurites *per* single cells. Besides, some cells even present a brush-like form as a sign of extensive microtubule-stabilization with growth cone-formation around the neuronal cell.

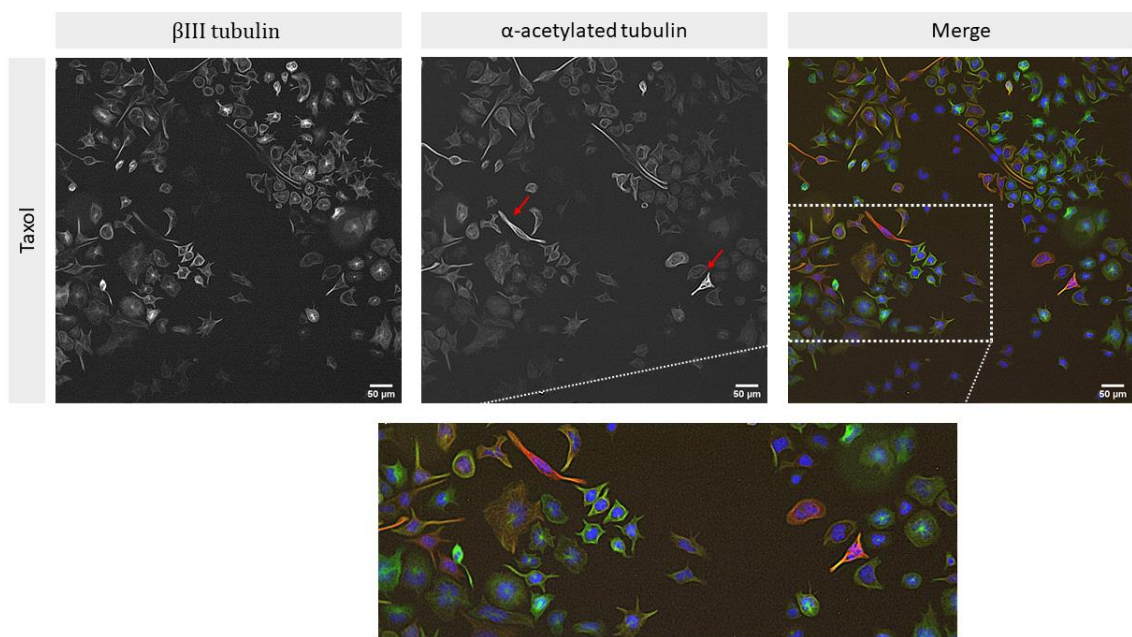


Figure 18. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at third day of induced differentiation and after 4h of 100nM Taxol treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

In order to quantify the microtubule morphological alterations caused by the different compounds in the ND7/23 cells the collar occupancy method developed by Boučanová et al. to distinguish oligodendrocytes differentiated stages were adapted. Image analysis was performed as described in Fig. 19.

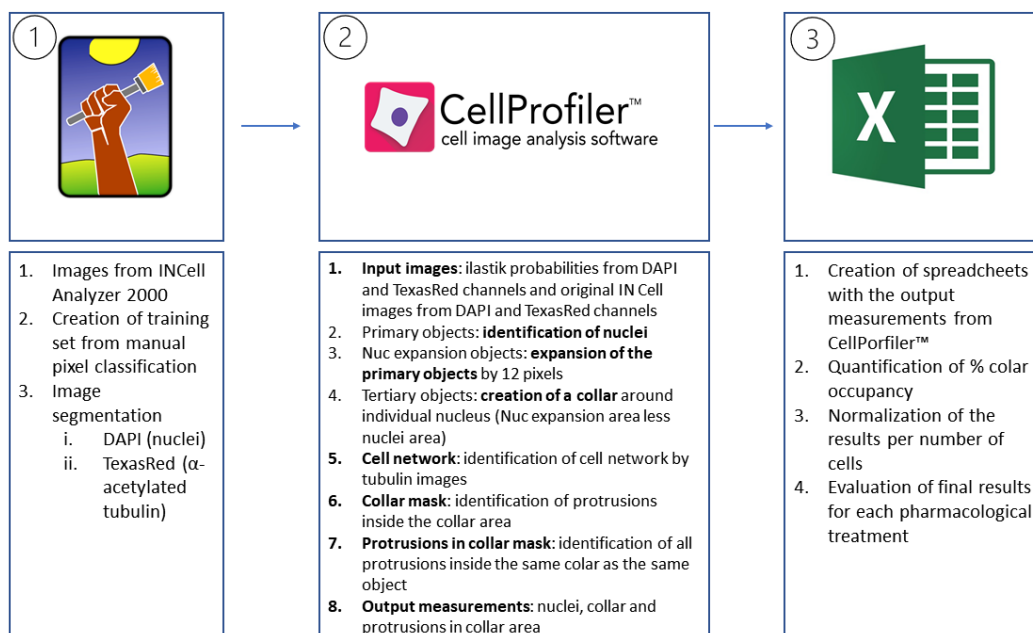


Figure 19. ND7/23 cell model image analysis process using collar occupancy method.

Briefly, nucleus and α -acetylated tubulin images were segmented using ilastik (Fig. 20) followed by further segmentation in CellProfiler™. There, the pipeline “Collar occupancy first identifies the nuclei objects and creates a 12-pixel radial expansion of each nucleus. The collar objects are created by the remotion of the nuclei to their expansion (Fig. 20). The tubulin network is identified by using the ilastik probabilities images of α -acetylated tubulin and then the collar and the tubulin objects are overlapped to identify the protrusions inside the same “collar” area. Finally, the software measures the area of each “Nuclei”, “Collar” and “protrusions in collar” objects, reporting all these values to a spreadsheet. To proceed with the analysis, it was used Microsoft Excel and calculated for each cell their percentage of collar occupancy ($\% \text{ collar occupancy} = \frac{\text{Area protrusions in collar}}{\text{Area collar}} \times 100$). Detailed explanations, accompanied with figures as examples, can be found in Appendix B.

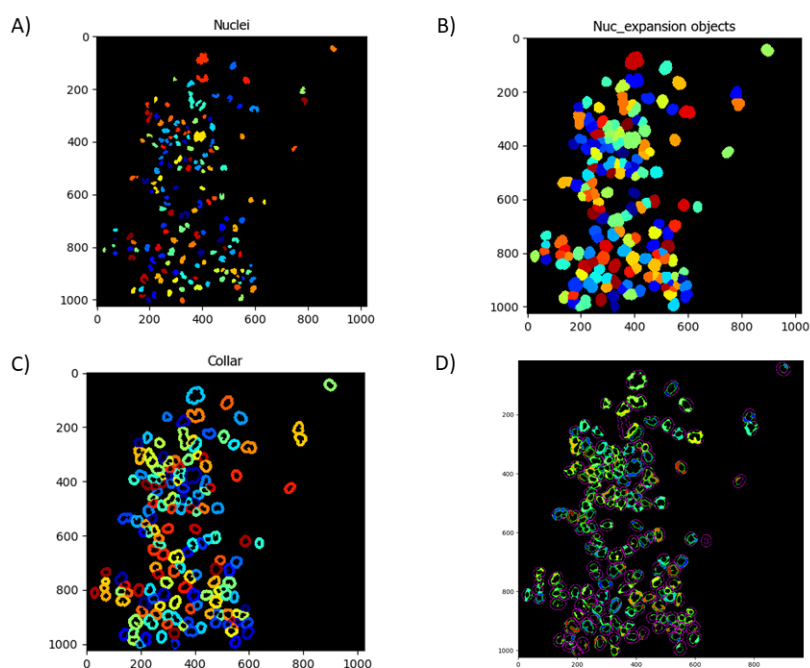


Figure 20. Representative images of outputs from “collar occupancy” pipeline in CellProfiler™ software. (A) Primary object “Nuclei”. (B) Secondary object “Nuc_expansion objects”. (C) Tertiary object “Collar”. (D) Identification of “Protrusions in collar”. Units are in pixel.

Once the referred compounds might have an impact on the cellular differentiation, cells were categorized into three different stages (Fig. 21).

Stage I includes the undifferentiated cells; stage II represents a mid-term between differentiated and undifferentiated cells; and stage III has the ND7/23 fully differentiated cells. By the collar occupancy method, the differentiated stage of a cell is proportional to its branching, and therefore to the percentage of the collar occupancy. Establishing ranges of percentages of collar occupancy for each stage of differentiated enables to separate undifferentiated from fully differentiated cells.

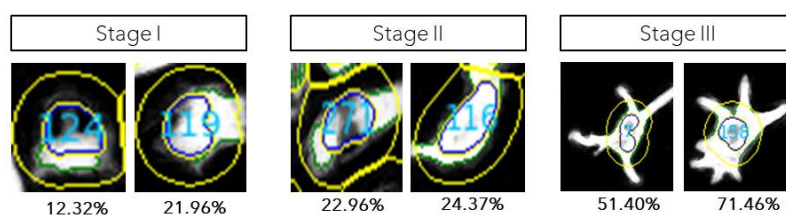


Figure 21. Representative examples of ND7/23 cells in undifferentiation/differentiation stages I-III, including the representation of “collar” (in yellow) and “nuclei” (in blue) objects. Stage I: undifferentiated cells (% collar occupancy range between 0% and 22,0%); Stage II: mid-term between differentiated and undifferentiated cells (% collar occupancy range between 22,1% and 24,5%); Stage III: fully differentiated cells (% collar occupancy range greater than or equal to 24,6%).

Since the differentiated cell phenotype observed after microtubule-destabilization with nocodazole was lost this pharmacological treatment was not used in the microtubule modulation quantification and only DMSO and Taxol conditions were analysed with the collar occupancy method.

Fig. 22 (A) display the percentage of cells in each differentiation stage for each condition. Stage I has in average a higher percentage of cells after DMSO treatment, while the trend reverses in stage III with taxol condition having a higher percentage of cells. Although the standard deviation in both stage I and III is high, this effect is observed in both drug treatments and can be related to the variation of cell differentiation achieved before the pharmacological treatment. Significant differences between DMSO and Taxol are more relevant in cells in Stage III. Since all conditions were subjected to the same differentiation protocol and were not expected that the drugs treatments had such sudden effects that would cause significant differences in cells in stage I and II. Therefore, in Fig. 22 (B) shows the frequency distribution in stage III for each treatment condition where all the cells represented are considered differentiated (with percentage of collar occupancy equal to or higher than 24,6%). Initial ranges with a lower percentage of collar occupancy have higher frequencies in DMSO condition but from 45% collar occupancy onwards the Taxol condition exhibits higher frequencies. The distribution in stage III translates in more branched cells, after taxol treatment, representing the effects of stabilization observed in ND7/23 cells phenotypes demonstrated in Fig. 18. The DMSO treatment has more cells that despite being differentiated have fine neurites, demonstrated in Fig. 16, represented by a lower percentage of collar occupancy.

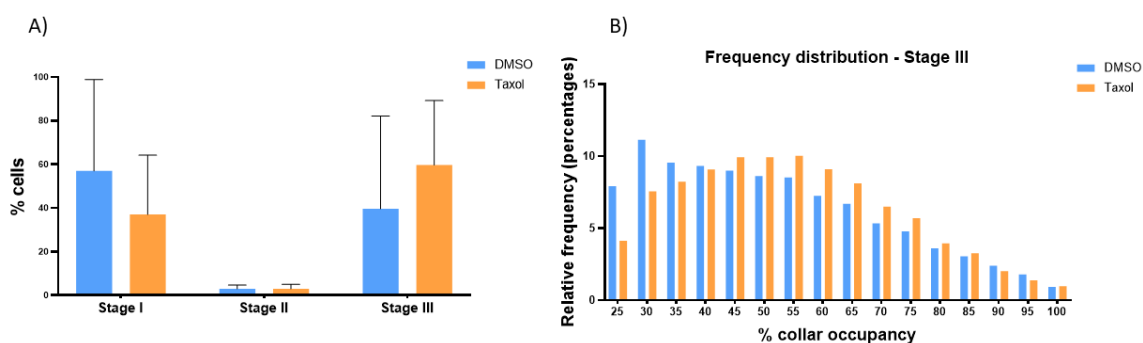


Figure 22. Quantification after control drugs treatment (DMSO and Taxol) using “Collar occupancy” method. A) Percentage (%) of cells per stage I, II, and III for DMSO and taxol conditions. B) Histogram showing the frequency distribution of % collar occupancy ranges in stage III for DMSO and taxol conditions. n=3 independent experiments, 2 replicates per condition and 49 fields per replicate. Graphs present Mean $\pm$ SD.

Based on these observations it was possible to conclude that ND7/23 differentiated cells model was successfully implemented as a tool for evaluation of microtubule-stabilization effects using morphology analysis and collar occupancy-based quantifications.

2.1.4. Evaluation of positive candidates for microtubule-stabilization in ND7/23 cell line model

The five positive candidates (Chapter III, section 2.2) selected as promising microtubule-stabilization agents were evaluated in the established ND7/23 cell line model. For that, each compound was incubated with the ND7/23 differentiated cells at a concentration of 100nM. For this preliminary study the same concentration used for Taxol was selected for the five potential hits. However, taking into consideration the higher stabilization effects observed in *U. maydis* model with the positive candidates comparing with the controls, the pharmacological treatment was analysed at three timepoints: 1h, 2h, and 4h.

The impact of the selected positive candidates on the MT stability was investigated following the tools established in section 2.1.3. and, therefore, cell morphology was evaluated by staining β III tubulin and α -acetylated tubulin. Fig. 22 show representative images of ND7/23 model drug treatment with Aprepitant, Atracurium besylate, Danazol, Disulfiram, and Pranlukast, respectively.

Aprepitant

Regarding investigation of Aprepitant on MT dynamics, (Fig. 23) at 1h time point, cells presented the typical neurites characteristic from their differentiated status. Additionally, it was visible that some cells also presented thicker protrusions when compared to the control. The elongated neurites morphology was maintained at 2h time point. However, at the same time neurons also presented the brush-like phenotype characteristic of microtubule-stability effect detected after 4h Taxol treatment. The higher incubation period (4h) with this drug did not induce any visible cytotoxicity. Moreover, it was clear that cells presented a novel microtubule hipper stabilization morphology, with extremely long thin neurites arising together Overall, pharmacological treatment with Aprepitant caused alterations in ND7/23 cells morphology indicative of MT stabilization, with this effect being higher for higher time points.

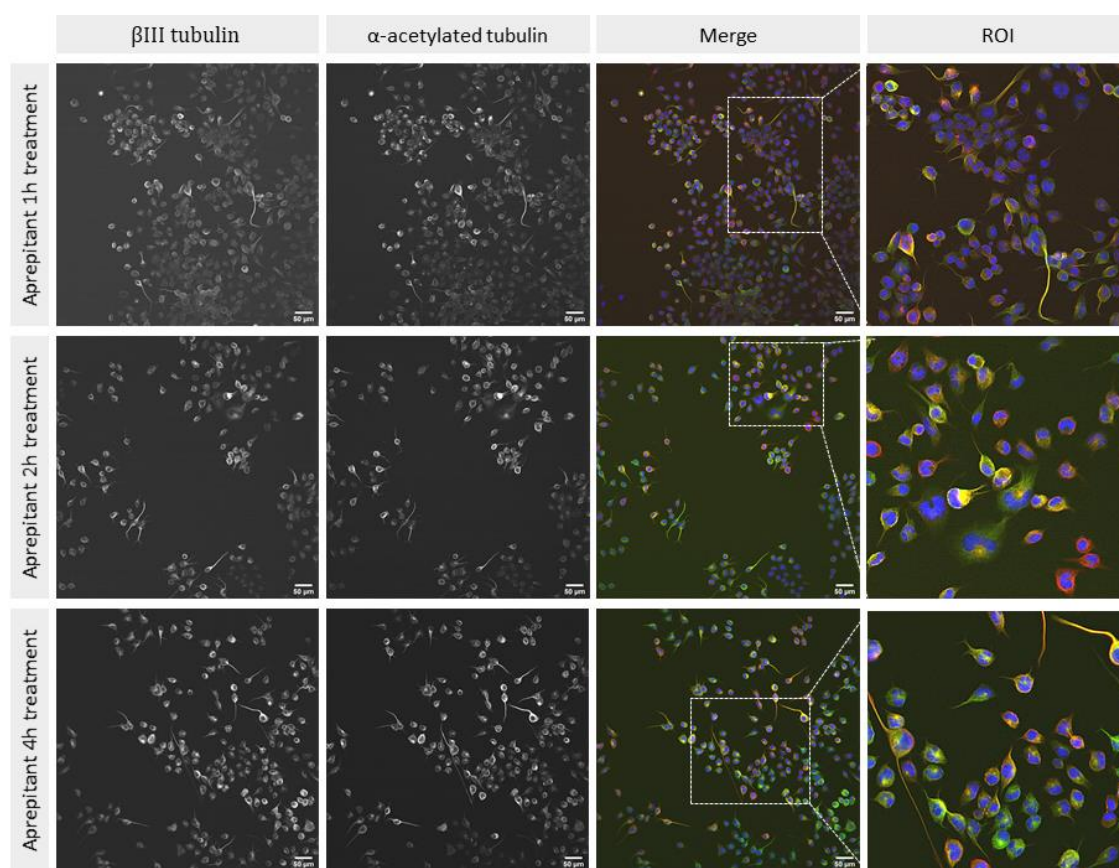


Figure 23. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at third day of induced differentiation and after 1h, 2h, and 4h 100nM Aprepitant treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

Fig. 24 shows the distribution of ND7/23 cells in stage III, given by the percentage of collar occupancy after Aprepitant treatment. Comparison of MT stability was firstly performed between Aprepitant different exposition times and 4h exposition to DMSO (Fig. 24 (A)). No clear

differences were observed in the frequency distribution of the treatment with Aprepitant for 1h and the treatment with DMSO, indicating that this drug does not have a prominent microtubule-stability effect on ND7/23 cells at earlier time points. This result corroborates the visual inspection above described for Fig. 23. Interestingly, a clear shift in cellular frequency distribution was observed for the treatment during 2h in comparison with DMSO control. The number of cells with higher percentages of collar occupancy was increased for the drug treatment, being the majority within 55% bin class, whereas for the control the majority were fitted around 40-45%. Although not depicted in the graph, the behaviour of neuronal cells treated for 2h with Aprepitant showed clear similarities when these cells were treated with Taxol (section 2.1.4). Finally, the treatment for 4h with Aprepitant revealed that the peak of collar occupancy was around 40%, a lower value when compared with 2h treatment. Nonetheless, one can also observe that the number of cells with high percentages of collar occupancy (class 95-100%) was pronouncedly higher for the treatment for 4h, either indicating that cells present thicker or a higher number of processes. Although quantitative analysis of the neurite length was not performed, visual inspection revealed that cells possessed extremely long neurites (Fig. 23), highlighting the strong effect of this drug in the stabilization of the neuronal MTs.

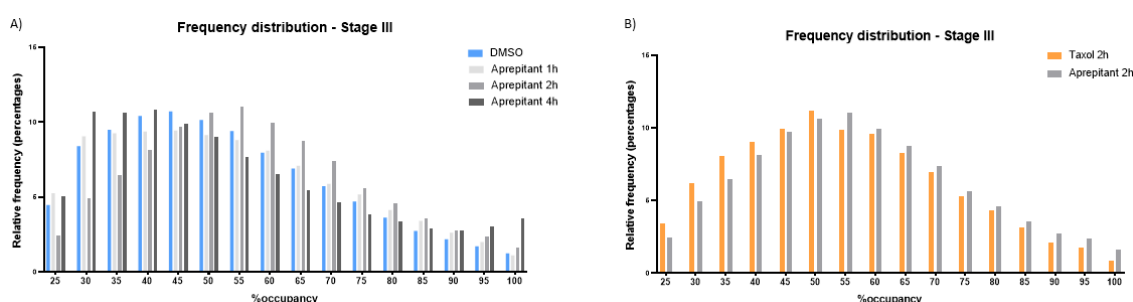


Figure 24. Distribution in stage III of quantification of % collar occupancy after Aprepitant treatment in ND7/23 cell line model and comparison with controls DMSO and Taxol treatments at day 3 post-differentiation. (A) % collar occupancy distribution after 1h, 2h, and 4h 100nM Aprepitant and 4h 1.0% DMSO treatments; (B) % collar occupancy distribution after 2h 100nM Aprepitant and Taxol treatments. For all graphs, blue bars refer to DMSO, orange bars refer to Taxol and grey bars refer to Aprepitant (lighter the colour, lower the time point for all conditions). n=2 independent experiments, 2 replicates per time point and 49 fields per replicate. Graphs present mean values.

Since cells after 2h of treatment with Aprepitant, in general revealed relatively higher percentages of collar occupancy, the stabilization of MTs was compared with the stabilizing agent Taxol. To investigate the MT stabilizing capacity of Aprepitant to the microtubule-stabilizer agent Taxol, the distribution of collar occupancy for cells in Stage III was compared for the 2h incubation time-point (Fig. 24 (B)). Curiously, the number of cells in higher percentages of collar occupancy was visibly higher for the Aprepitant treatment. These results indicate that Aprepitant treatment in the ND7/23 cells model has a clear microtubule-stabilizing action with preliminary data pointing to a stronger stabilization effect than the Taxol.

Atracurium besylate

MT stability assessment of Atracurium besylate candidate based on cell morphology evaluation is demonstrated in Fig. 25. After 1h drug treatment the ND7/23 cells neurites remained similar to what was observed after DMSO treatment. However, when exposed during 2h an alteration

in morphology with a considerable number of cells acquiring a brush-like phenotype was observed. At the same time other cells presented thick neurites and a denser tubulin network around the cellular body, indicating cytoskeleton retraction caused by the stability of the MTs. After 4h Atracurium besylate treatment with a considerable number of cells with brush-like morphology and elongated and thicker neurites. It is important to mention that it was also visible the presence of a retraction bulb-like formation in one neurite (highlighted in the region of interest (ROI)), which indicates initial steps of MT destabilization. This might be due to an excessive MT stability caused by Atracurium besylate during 4h.

Fig. 26 shows the distribution of collar occupancy in Stage III of ND7/23 cells after Atracurium besylate treatment. After 1h of treatment, no differences were seen between the drug treatment and the DMSO control (Fig. 26 (A)), where the majority of the cells have 30-45% of collar occupancy in both conditions. Surprisingly, a high number of cells were also found within the 90-100% range. These variations were not expected considering the similar cell morphologies observed in these conditions. One should however note that, some problems could have arisen from the cell segmentation in ilastik, where the probabilities map might be overestimated. Nonetheless, to corroborate these results more independent experiments and a manual validation of the algorithm should be performed.

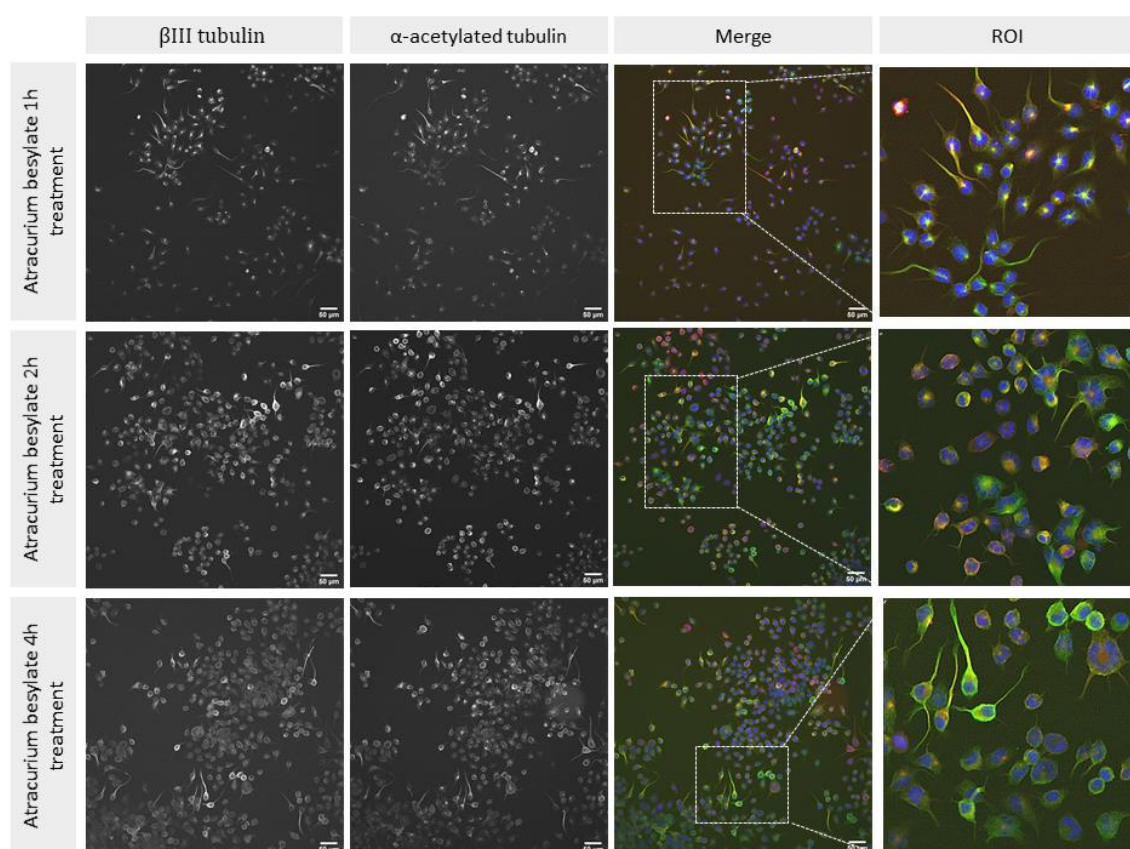


Figure 25. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at third day of induced differentiation and after 1h, 2h, and 4h 100nM Atracurium besylate treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

Contrary, for higher exposure times (2h and 4h) to Atracurium besylate, an evident shift in frequency distribution for higher percentages of collar occupancy was observed. For both time points, the majority of the cells were found in the 55-60% range. Interestingly, the number of

cells for higher percentages of collar occupancy (90-100%) were tendentially decreased for both time points, being more notable for the 4h treatment. As highlighted before, this tendency might be due to the initial steps of MT stabilization detected in neurons which are consequently translated into a reduction of the number of cells with high percentages of collar occupancies.

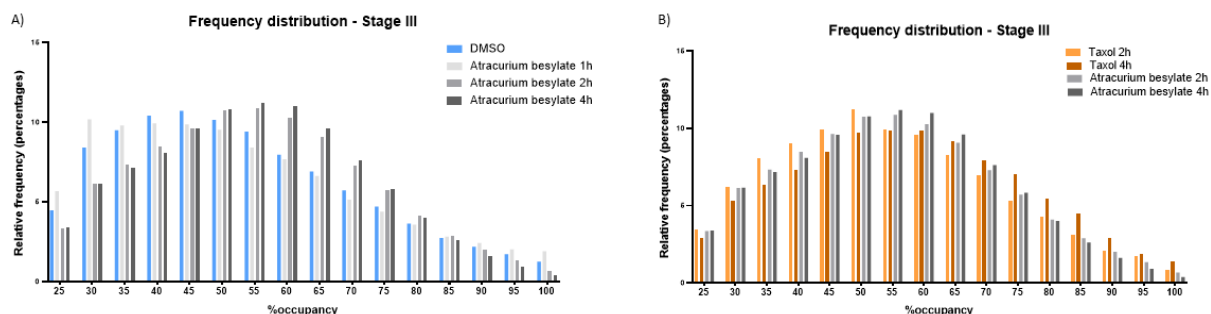


Figure 26. Distribution in stage III of quantification of % collar occupancy after Atracurium besylate treatment in ND7/23 cell line model and comparison with controls DMSO and Taxol treatments at day 3 post-differentiation. (A) % collar occupancy distribution after 1h, 2h, and 4h 100nM Atracurium besylate and 4h 1.0% DMSO treatments; (B) % collar occupancy distribution after 2h and 4h 100nM Atracurium besylate and Taxol treatments. For all graphs, blue bars refer to DMSO, orange bars refer to Taxol and grey bars refer to Atracurium besylate (lighter the colour, lower the time point for all conditions). n=2 independent experiments, 2 replicates per time point and 49 fields per replicate. Graphs present mean values.

A closer look to investigate the MT-stabilization effect of Atracurium besylate in the ND7/23 cell line model is presented in Fig. 26 (B) where cells treated for 2 and 4h with the positive candidate and the Taxol control are compared.

Overall, it is possible to consider that Atracurium besylate has an undeniable microtubule-stabilizing effect on ND7/23 cells. This preliminary analysis reveals a maximum effect at 2h treatment similar to the stabilization levels observed for Taxol. Nevertheless, Atracurium besylate effects were decreased for the 4h treatment, which was previously described as the ideal time point for Taxol. This indicates that more studies should be conducted in order to optimize the therapeutic effect of this positive candidate.

Danazol

The assessment of MT-stability capacity from Danazol treatment was first based on qualitative cell morphology analysis, illustrated in Fig. 27. After 1h of drug treatment thin neurites could be observed in the ND7/23 cells and no visible signs of altered neuronal morphology were seen. However, branching in neurite ends and small elongation of the tubulin network in the cellular body was observed in a few cells. When exposed during 2h treatment, alterations in morphology were observed with some cells acquiring a brush-like morphology and others a small and thin neurite. After 4h Danazol treatment the majority of the cells lost their neurites, acquiring a rounded phenotype with a thin dense layer of tubulin around the nucleus. Considering the inconsistent cell morphologies throughout the treatment, the destruction of neurites might not be associated with an excess of MT-stability but some cytotoxicity since, based on these preliminary results, it was not possible to conclude about MT-stability capacity of Danazol.

Fig. 28 shows the distribution of collar occupancy in stage III of ND7/23 cells after Danazol treatment. After 1 and 2h of treatment, a slight shift in frequency distribution for lower percentages of collar occupancy was observed. For these time points the majority of the cells

were found in the 25-40% range, lower ranges than comparing with DMSO control. However, after 2h of treatment a higher number of cells was also found within the 55-95% range. These results were not expected considering the cell morphologies observed in these conditions. Nevertheless, a closer look at both results points to fact that the higher level of neurites branched detected after 1h Danazol treatment are not quantified by the “collar occupancy” method since the ramifications are in the ends of the protrusions and are not detected inside the “Collar” objects. The variations showed for 2h of treatment translate the 2 different neuron morphologies observed for this time-point representative of: i) a few cells with a brush-like cytoskeleton; and ii) other cells with a similar morphology to DMSO control. In accordance with the cell morphology observed after 4h of treatment, the higher number of cells were found in the 35-40% range, a shift to lower percentages of collar occupancy than DMSO control.

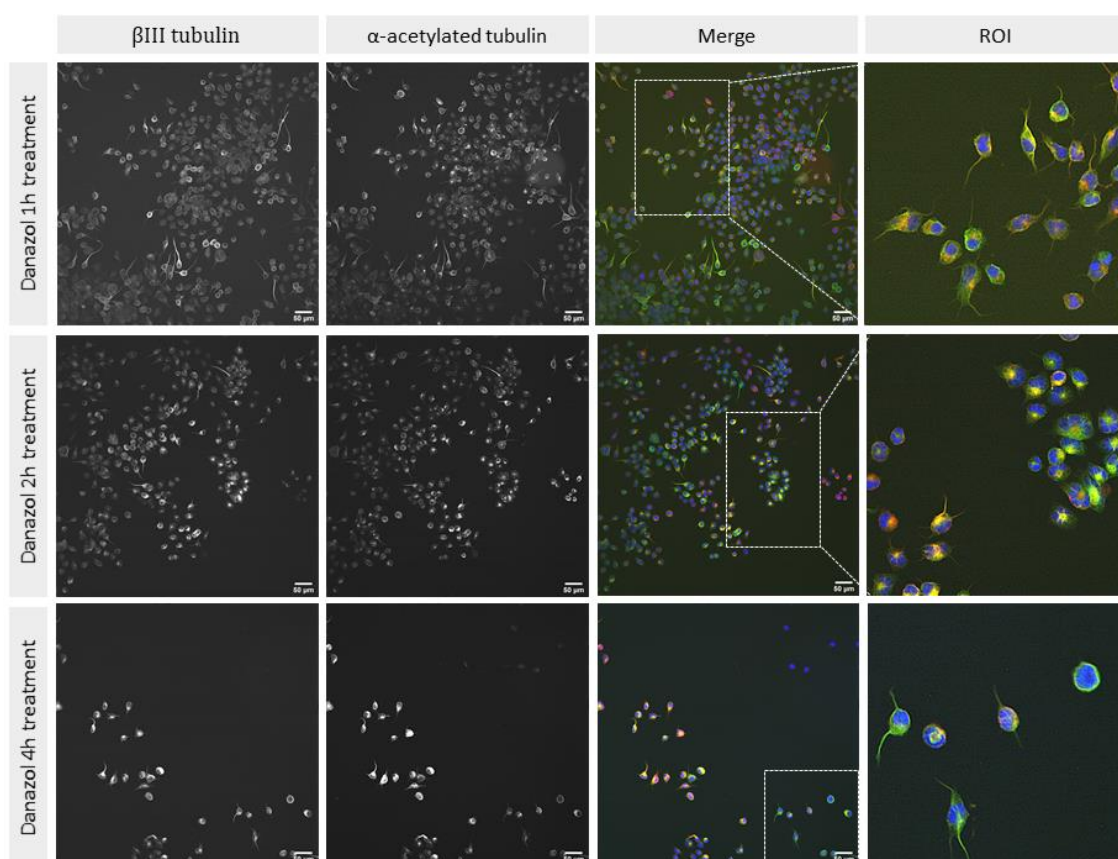


Figure 27. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at the third day of induced differentiation and after 1h, 2h, and 4h 100nM Danazol treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

Since cells after 2h of treatment with Danazol, in general, revealed higher percentages of collar occupancy, the stabilization of MTs was compared with the stabilizing agent Taxol (Fig. 28 (B)). After 2h of treatment an increased number of cells were found within the 25-35% range. Surprisingly, a higher number of cells were also found within the 90-100% range. These variations may be once again the translation of the 2 different cell morphologies observed after 2h of Danazol treatment. Gathering all the results from Danazol treatment in the ND7/23 cells model at this point it is not possible to completely conclude that Danazol has a microtubule-stabilization effect.

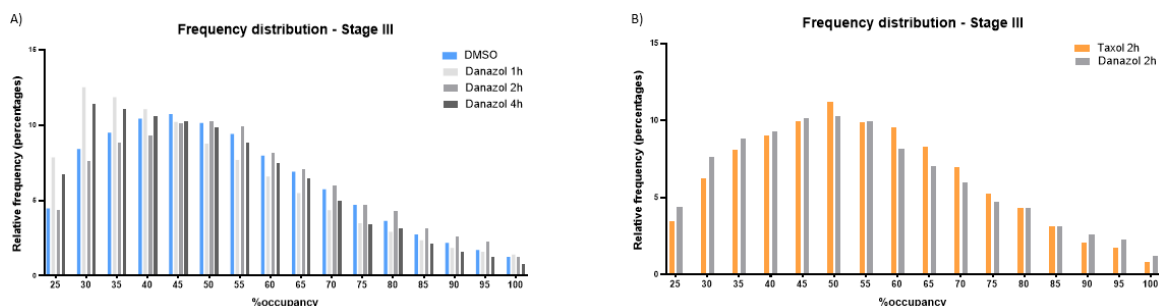


Figure 28. Distribution in stage III of quantification of % collar occupancy after Danazol treatment in ND7/23 cell line model and comparison with controls DMSO and Taxol treatments at day 3 post-differentiation. (A) % collar occupancy distribution after 1h, 2h, and 4h 100nM Danazol and 4h 1.0% DMSO treatments; (B) % collar occupancy distribution after 2h 100nM Danazol and Taxol treatments. For all graphs, blue bars refer to DMSO, orange bars refer to Taxol and grey bars refer to Danazol (lighter the colour, lower the time point for all conditions). n=2 independent experiments, 2 replicates per time point and 49 fields per replicate. Graphs present mean values.

Disulfiram

MT stability assessment of Disulfiram candidate based on qualitative cell morphology evaluation is demonstrated in Fig. 29. After 1 and 2h of treatment, some cells presented thin and elongated neurites and others also endue a tight and constrained tubulin network, signs of a clear MT stability. However, at the 2h time-point a few cells acquired a rounded form, demonstrating signs of excess of MT stability. After 4h of treatment a higher number of cells demonstrated the loss of neurites acquiring a rounded form. Surprisingly, a few cells seem to recover from the MT-destabilized phenotype and demonstrated thick neurites.

Fig. 30 shows the percentage of collar occupancy distribution in stage III after Disulfiram treatment comparing with DMSO control. After 1h and 2h of treatment no differences were seen between the drug treatment and the DMSO control (Fig. 30 (A)), where the majority of the cells have 30-45% of collar occupancy in both conditions. Surprisingly, a high number of cells were also found within the 95-100% range. These variations were not expected considering the similar morphologies observed in these conditions. To be noted that the extended neurites presented after Disulfiram treatment, and absent after DMSO, can not be quantified with the “collar occupancy” method. One should however consider that, problems could have arisen from the cell segmentation in ilastik, where the probabilities map might be overestimated. To corroborate these results further experiments should be performed. After 4h of treatment, a higher number of cells were found within the 55-85% range of collar occupancy. This result correlate with the morphology alterations reported after 4h Disulfiram treatment: i) the microtubule-stabilization effect caused neurites growth, being responsible for higher relative frequencies in 55-85% of collar occupancy; and ii) the lower relative frequencies in 90-100% of collar occupancy might be due to an excess of stability and not to an absence of MT modulation.

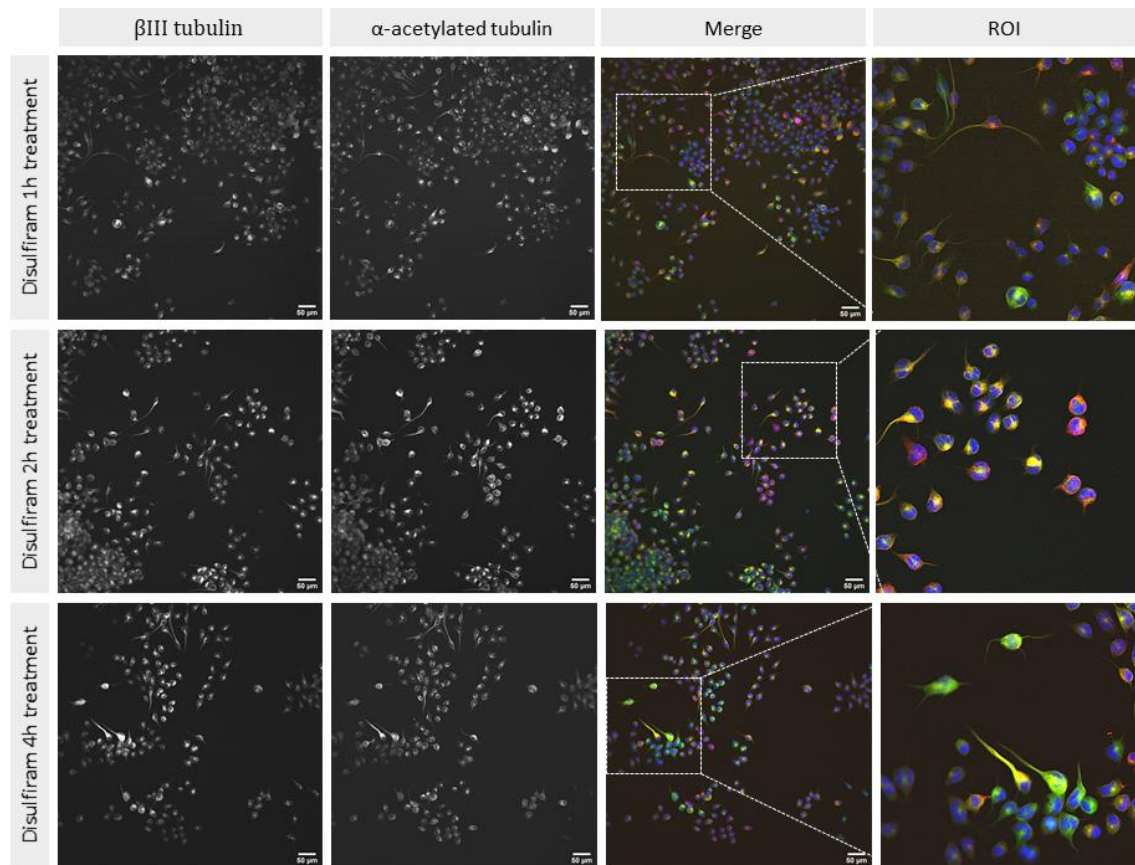


Figure 29. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at third day of induced differentiation and after 1h, 2h, and 4h 100nM Disulfiram treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

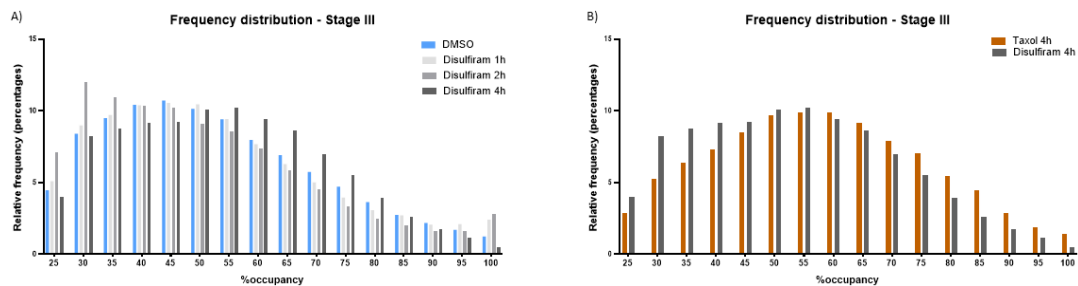


Figure 30. Distribution in stage III of quantification of % collar occupancy after Disulfiram treatment in ND7/23 cell line model and comparison with controls DMSO and Taxol treatments at day 3 post-differentiation. (A) % collar occupancy distribution after 1h, 2h, and 4h 100nM Disulfiram and 4h 1.0% DMSO treatments; (B) % collar occupancy distribution after 2h 100nM Disulfiram and Taxol treatments. For all graphs, blue bars refer to DMSO, orange bars refer to Taxol and grey bars refer to Disulfiram (lighter the colour, lower the time point for all conditions). n=2 independent experiments, 2 replicates per time point and 49 fields per replicate. Graphs present mean values.

Overall, it is possible to consider Disulfiram as capable of a microtubule-stabilizing effect on ND7/23 cells. This preliminary analysis reveals a maximum effect at 4h treatment. Hence, this condition was analysed in comparison with 4h Taxol treatment (Fig. 30 (B)). The higher number of cells after Taxol treatment was within 60-100% and after Disulfiram treatment was within 25-45% of collar occupancy, which indicates that Disulfiram might have a lower MT-stabilizing effect than Taxol, but further studies should be conducted.

Pranlukast

Lastly, it was investigated the MT-stability capacity of Pranlukast compound. In Fig. 31 are analysed the cell morphology after drug treatment in the ND7/23 cells. After 1, 2 and 4h of treatment single cells presented an elevated degree of branching with more than one neurite. It is important to mention that it was also visible the presence of cells with brush-like morphology. Surprisingly, in longer incubation periods the ND7/23 cells do not demonstrate signs of excess of MT-stability, early indications that Pranlukast has the capacity to be a microtubule-stability agent.

Fig. 32 shows the distribution of collar occupancy in stage III of ND7/23 cells after Pranlukast treatment. In the three time points showed a similar behaviour regarding the frequency distribution when compared with DMSO treatment (Fig. 32 (A)). The majority of the cells had 65-100% of collar occupancy in the three conditions, which correlates with a higher number of cells displaying microtubule-stability morphologies. The considerable difference observed between Pranlukast treatment and DMSO in the ND7/23 cells points to the idea that Pranlukast induces microtubule-stability effect.

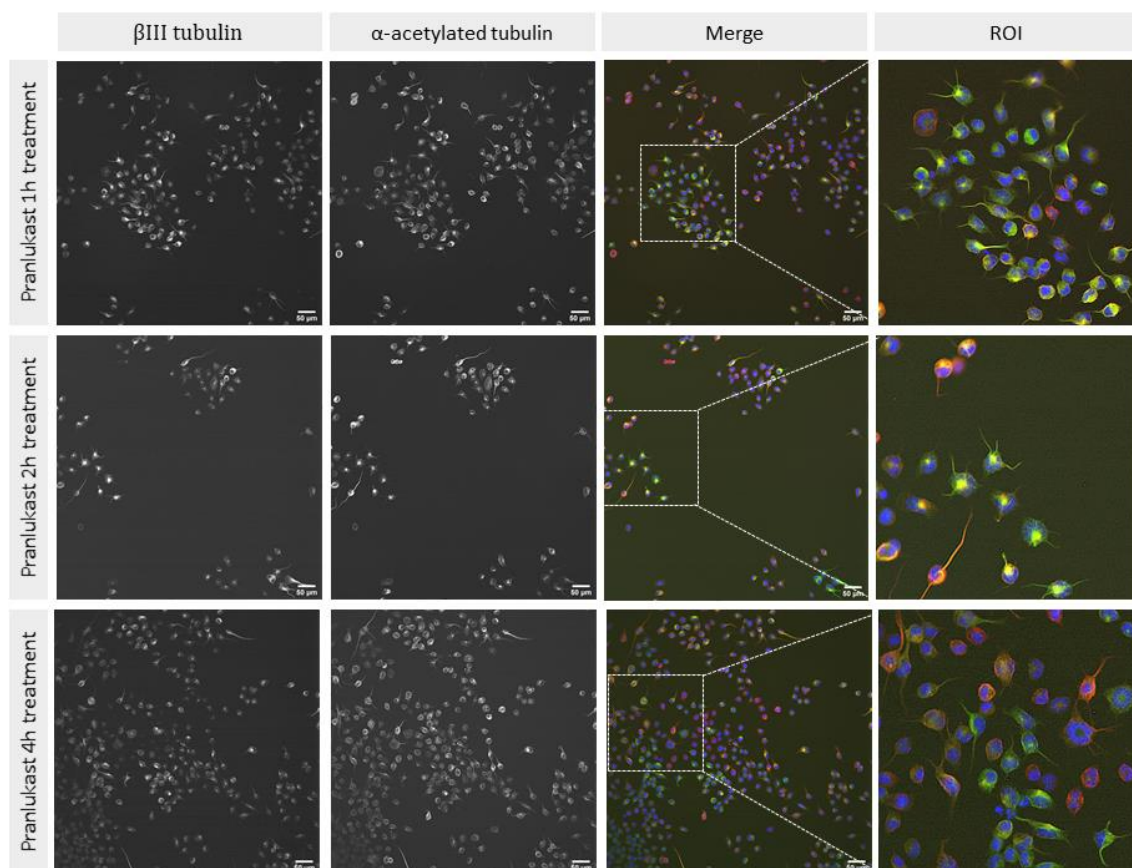


Figure 31. Representative images of acquired channels in IN Cell Analyzer 2000 of ND7/23 cell model at the third day of induced differentiation and after 1h, 2h, and 4h 100nM Pranlukast treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

After all the time-points tested for Pranlukast, cells revealed high percentages of collar occupancy, the stabilization of MTs was compared with Taxol (Fig. 32 (B)). At the same time point, Pranlukast had a higher number of cells within the 70-100% range of collar occupancy, which correlates with a higher number of cells displaying microtubule-stability morphologies.

Overall, it was possible to consider that in the ND7/23 cells model the Pranlukast compound had a microtubule-stability effect. The preliminary analysis reveals higher levels of stabilization than observed for Taxol.

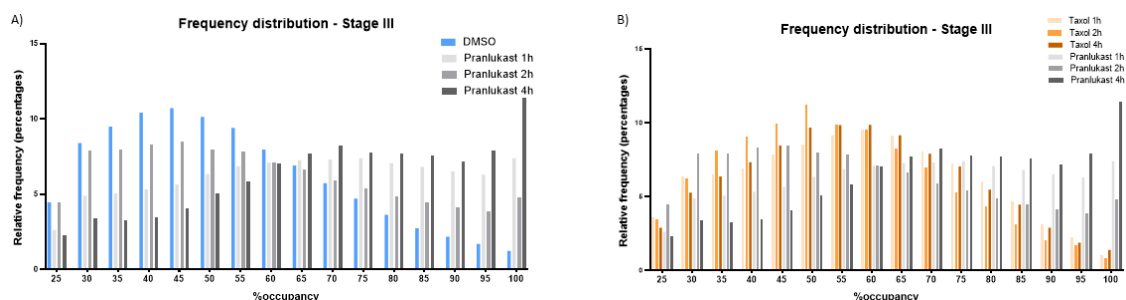


Figure 32. Distribution in stage III of quantification of % collar occupancy after Pranlukast treatment in ND7/23 cell line model and comparison with controls DMSO and Taxol treatments at day 3 post-differentiation. (A) % collar occupancy distribution after 1h, 2h, and 4h 100nM Pranlukast and 4h 1.0% DMSO treatments; (B) % collar occupancy distribution after 2h 100nM Pranlukast and Taxol treatments. For all graphs, blue bars refer to DMSO, orange bars refer to Taxol and grey bars refer to Pranlukast (lighter the colour, lower the time point for all conditions). n=2 independent experiments, 2 replicates per time point and 49 fields per replicate. Graphs present mean values.

2.1.5. Cellular metabolic evaluation after drug treatment – Intracellular [Ca^{2+}] measurements

In the neuron's interaction with the environment, and in the complex cytoskeleton network, the calcium ions (Ca^{2+}) act as a universal second messenger that transduce signals from upstream events. Indeed, on many occasions' pharmacological molecules effects on their targets is translated in signals through Ca^{2+} messenger. Thereby, the Ca^{2+} signal is often studied in drug evaluation on a functional level.

To regulate MT dynamics their +TIPs interact with different cytoplasmic protein such as Ca^{2+} - and calmodulin-regulated MAP. Thus, a preliminary evaluation of the selected microtubule-stabilization positive candidates was performed by assessing alterations in intracellular Ca^{2+} using Fluo-4 AM assay after drug treatment.

At day 3 post-differentiation ND7/23 cells were subjected either one of the drug treatments: i) 4h 1.0% DMSO; ii) 4h 1 μ M Nocodazole; iii) 1h, 2h, and 4h 100nM Taxol; iv) 1h, 2h, and 4h 100nM Aprepitant; v) 1h, 2h, and 4h 100nM Atracurium besylate; vi) 1h, 2h, and 4h 100nM Danazol; vii) 1h, 2h, and 4h 100nM Disulfiram; viii) 1h, 2h, and 4h 100nM Pranlukast. After, the medium was removed and a working solution of Fluo-4 AM was added to 2 wells per condition and HBSS was added to one well per condition (to serve as a negative control in the Fluo-4 AM assay). After the incubation period, the fluorescence was measure with excitation at 485 nm and emission at 520 nm.

It was expected that upon MT-stabilization calcium oscillations occur and so, higher intracellular [Ca^{2+}], which would translate into higher absorbance values [213]. Fig. 33 displays fluorescence measurements for each condition normalized by fluorescence values of HBSS of each corresponding condition.

Nocodazole treatment revealed an expected lower intracellular [Ca^{2+}] than DMSO and Taxol at 1h and 4h treatment (Fig. 33 (A)), considering the destabilization effect of Nocodazole. MT-

stabilization effect of Taxol cause calcium oscillations which were detected when comparing with Nocodazole fluorescence. Higher values of Ca^{2+} were expected after Taxol treatment when compared to DMSO. Surprisingly, they displayed similar fluorescence values (Fig. 33 (A)). Ca^{2+} is a universal messenger used in numerous mechanisms and DMSO can be causing cascade of events in cells that justify the fluorescence numerous similar to Taxol [213]. Contrary to the expected, Taxol treatment after 2h displayed fluorescence values lower than the 4h and 1h treatment (Fig. 33 (A)). However, it should be pointed out the high standard deviation observed in these time points.

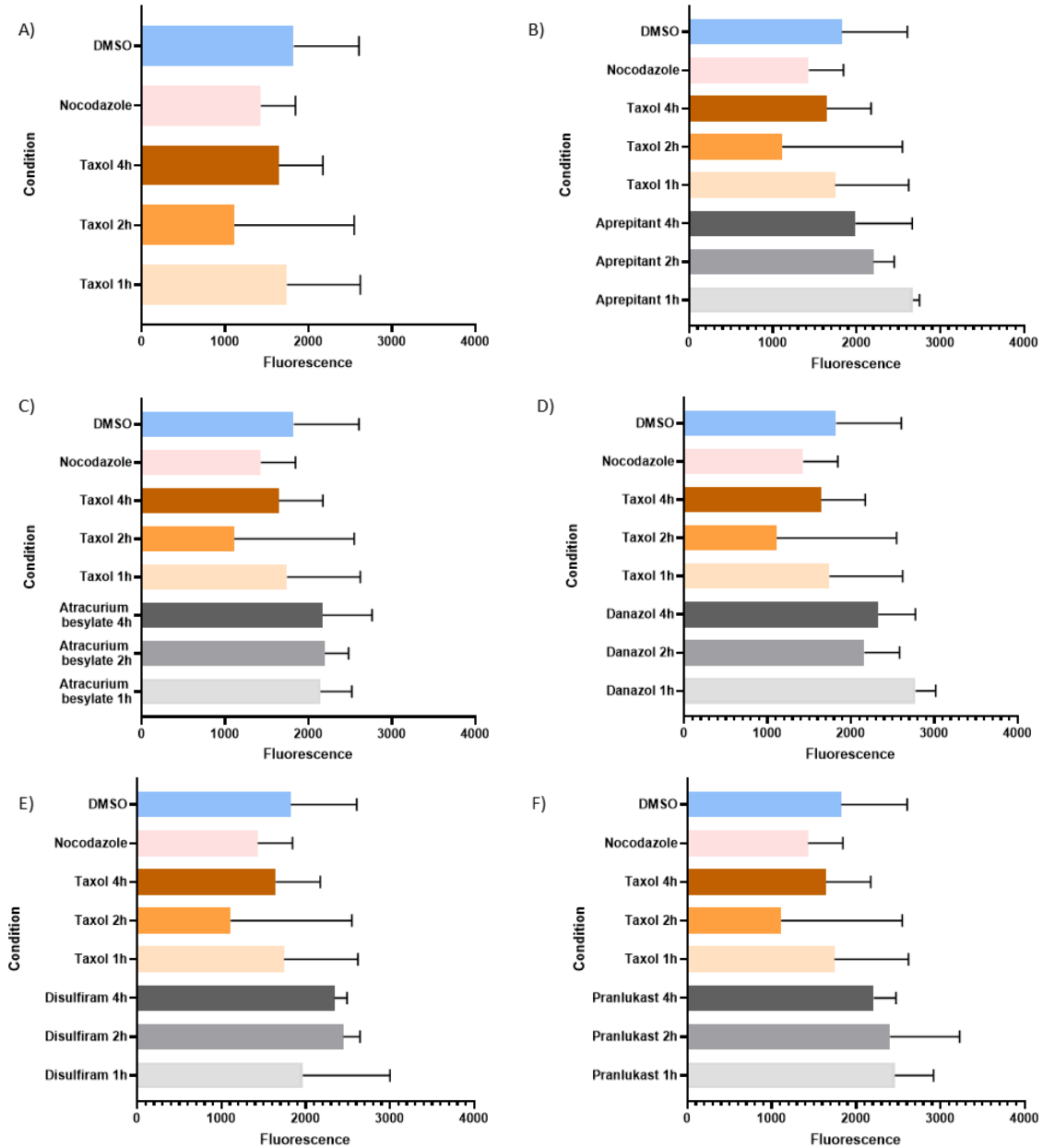


Figure 33. Fluorescence measurements normalized by fluorescence values in HBSS of each condition. A) After drug-control treatment of 1.0% DMSO, 1 μ M Nocodazole and 100nM Taxol. A); B) After 100nM Aprepitant 1h, 2h, and 4h treatment comparing with drug-controls; C) After 100nM Atracurium besylate 1h, 2h, and 4h treatment comparing with drug-controls; D) After 100nM Danazol 1h, 2h, and 4h treatment comparing with drug-controls; E) After 100nM Disulfiram 1h, 2h, and 4h treatment comparing with drug-controls; F) After 100nM Pranlukast 1h, 2h, and 4h treatment comparing with drug-controls. n=3 independent experiments. Graphs present Mean $\pm$ SD.

After establishing the method with differentiated ND7/23 cells, the aim was to evaluate the impact of the candidate drugs in intracellular Ca^{2+} . The controls (DMSO, Nocodazole and Taxol) were compared with the fluorescence values measured after treatment with the positive candidates. Aprepitant treatment displayed a fluorescence higher than the controls after 1h and 2h treatment and similar results to DMSO and Taxol after 4h treatment (Fig. 33 (B)). These results point to high intracellular calcium oscillations in cells after Aprepitant treatment, indicating MT stabilizing effect, with 1h and 2h treatment, showing a stronger effect than Taxol.

Regarding Atracurium besylate treatment (Fig. 33 (C)) all the time points displayed similar fluorescence measurements, higher than DMSO and Taxol, which points to a MT modulation effect.

Danazol, as in Taxol treatment, at 1h and 4h time points had higher fluorescence values. However, after 2h lower intracellular $[\text{Ca}^{2+}]$ (Fig. 33 (C)) were observed. Interestingly, Danazol also had higher absorbance values than DMSO and Taxol, a preliminary indication of microtubule-stability effect.

Different from the drugs behaviour demonstrated by the other candidates, Disulfiram had lower fluorescence values at 1h time point than at 2h and 4h treatment (Fig. 33 (D)). However, at 1h time point it had a fluorescence value similar to DMSO. Curiously, 2h and 4h after Disulfiram treatment the intracellular Ca^{2+} values were higher than with Taxol. These results imply that Disulfiram might have a MT-stability effect at earlier time points.

Finally, Pranlukast also displayed higher fluorescence values than the controls (Fig. 33 (E)), although at the longer time point of 4h it was detected a downward trend of intracellular $[\text{Ca}^{2+}]$, perhaps due to excess of MT-stability effect after 4h treatment.

The five selected positive candidates revealed functionally a preliminary indication of microtubule-stabilization effect after treatment in the ND7/23 cell line.

2.2. Mouse primary cortical neurons: a tool to validate microtubule-stabilizing compound-candidates

2.2.1. Assessment of drug-controls for microtubule-stabilizing agents in primary cortical neurons model: DMSO, Nocodazole and Taxol

In the same line of what was developed for the ND7/23 cells model, a mouse primary cortical neurons were also established as a tool in the evaluation of microtubule-stabilizing agents. Workflow described in Fig. 34.

Verstraelen et al. [213] described an optimal cell density of primary cortical neurons *in vitro* of 78.900 cells/cm². Nevertheless, this higher density was too high for our *in vitro* model and so, lower cell densities were evaluated (39.000 cells/cm² and 26.300 cells/cm²). Cells were treated with the control-drugs DMSO, Nocodazole and Taxol (described in Chapter IV, section 2.1.3.) and their morphology assessed for both densities.

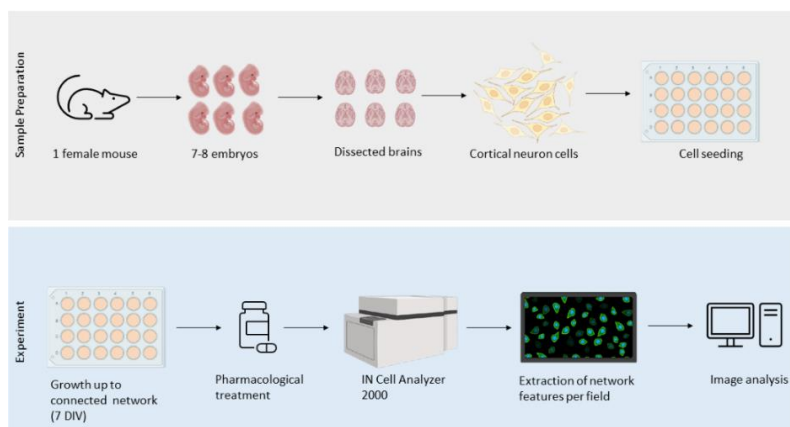


Figure 34. Schematic representation of the workflow followed for the establishment of mouse primary cortical neurons as a model for evaluation of microtubule-stabilization effect.

At DIV 7 primary cortical neurons were subjected to the pharmacological treatment for 4h with 1.0% DMSO, 1 μ M Nocodazole and 100nM Taxol. Neuronal morphology was evaluated by staining β III tubulin and α -acetylated tubulin as previously described. Fig. 35-37 show representative images of mouse primary cortical neurons model for DMSO, Nocodazole, and Taxol (26.300 and 39.500 cells/cm²), respectively.

Although DMSO control was established in the same conditions used in the ND7/23 cell line model, primary cells can be more sensitive to the toxic DMSO effect. However, as shown in Fig. 35, at both cell densities of 26.300 and 39.500 cells/cm² the primary cortical neurons displayed an expected morphology with extended neurites starting to form networks at lower seeding density and already showing a well-established network in the 39.500 cells/cm² seeding density.

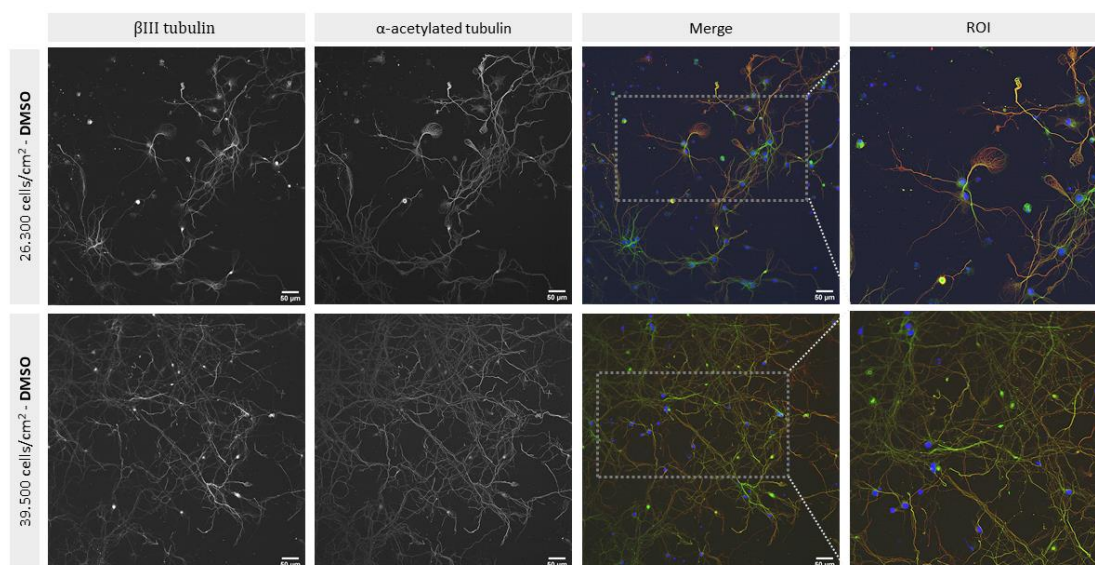


Figure 35. Representative images of acquired channels in IN Cell Analyzer 2000 of 26.300 and 39.500 cells/cm² of mouse primary cortical neurons model at 7 DIV and after 4h 1.0% DMSO treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

In the analysis of the negative control (Fig. 36) established with the antimitotic Nocodazole it was possible to see destabilization of β III tubulin, by the loss of the cellular integrity, visible in

both cell densities. Furthermore, the intense dots (ROI Fig. 36) detected in the tubulin network are a characteristic phenotype of Wallerian degeneration (WD), a tightly regulated form of axon loss [220, 221]. On the other hand, the maintenance of α -acetylated tubulin even after 4h of Nocodazole treatment was expected since this tubulin is less prone to destabilization [213].

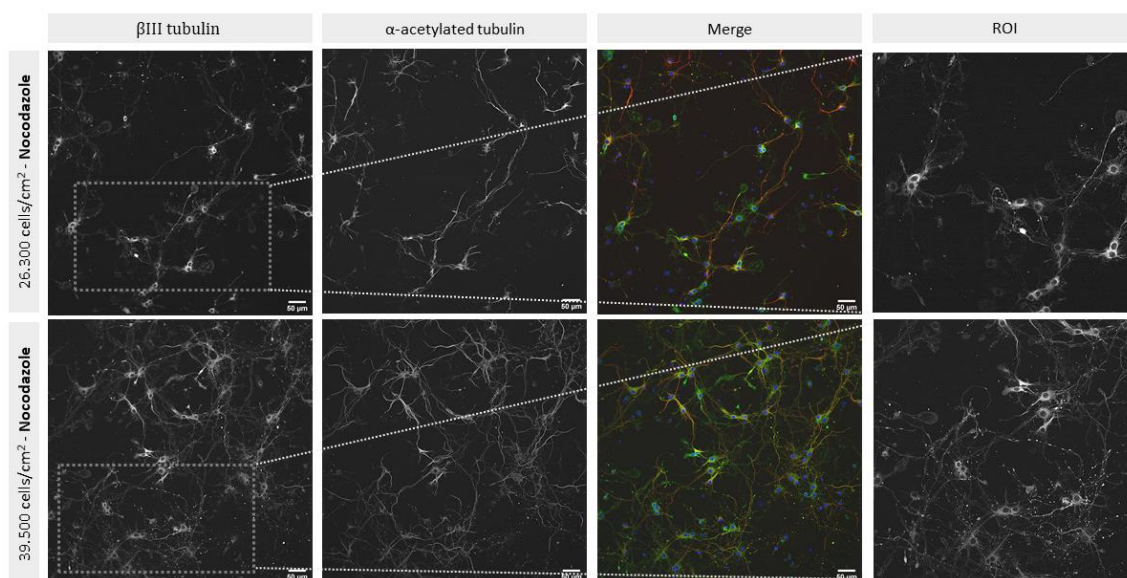


Figure 36. Representative images of acquired channels in IN Cell Analyzer 2000 of 26.300 and 39.500 cells/cm² of mouse primary cortical neurons model at 7 DIV and after 4h 1 μ M Nocodazole. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

The effects induced after 4h of pharmacological treatment with the MT-stabilizer Taxol in primary cortical neurons (Fig. 37) are shown by peaks of intensity in both types of tubulins analysed (observed in the two cell seeding densities). Additionally, the formation of growth cones (highlighted by red arrow) is a clear indication of MT-stabilization.

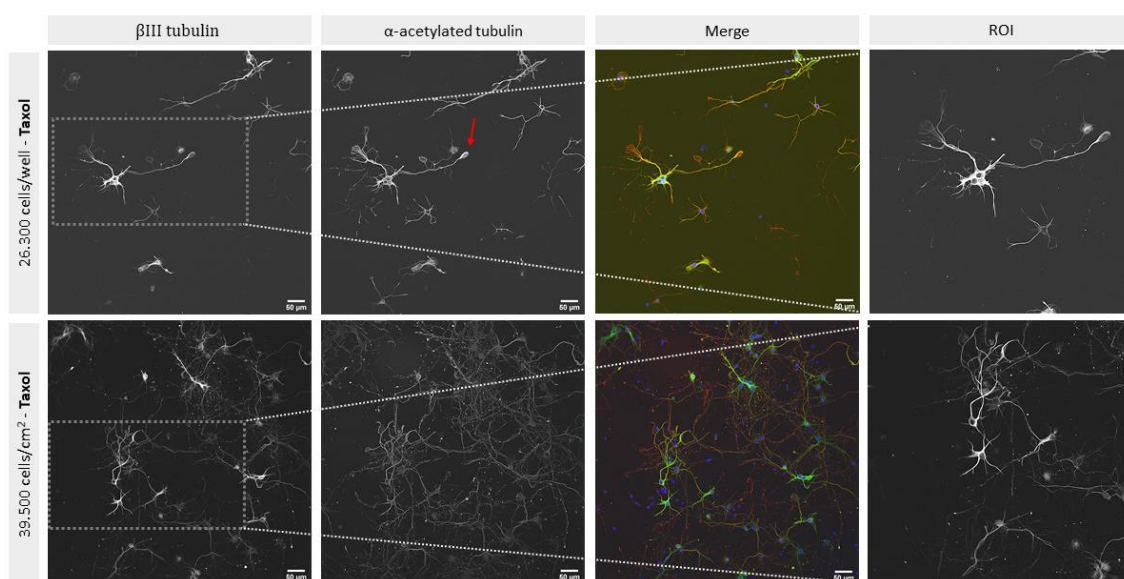


Figure 37. Representative images of acquired channels in IN Cell Analyzer 2000 of 26.300 and 39.500 cells/cm² of mouse primary cortical neurons model at 7 DIV and after 4h 100nM Taxol. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

Each drug-control showed the expected effects in MT-dynamics of primary cortical neurons, but it was noted an alteration in the cell viability after the treatments. Indeed, a closer look in the image analysis demonstrate two distinct nuclei. As shown in Fig. 38, while a few nuclei are bulky with small intense dots representative of nucleoli (highlighted by green arrows), other are small, constrained nucleus (highlighted by orange arrows) usually associated with apoptotic cells. Therefore, it was important to not only developed an image quantification that translate the stability/destabilization effects that occurred in the tubulin cytoskeleton of the primary cortical neurons but also quantifies the percentage of live/dead cells, which would allow a deeper analyse regarding possible toxic action on the part of pharmacological treatment.

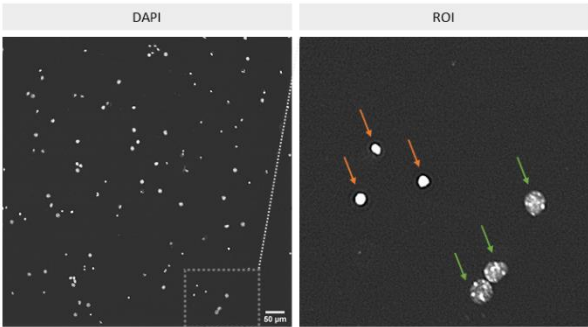


Figure 38. Representative images of mouse primary cortical neurons nucleus stained with Hoechst. Green arrows highlight live cells and orange arrows highlight apoptotic cells. Scale bar: 50μm

The image analysis for MT-stability quantification in primary cortical neurons was based on the measurement of the mean tubulin cytoskeleton length and is summarily presented in Fig. 39.

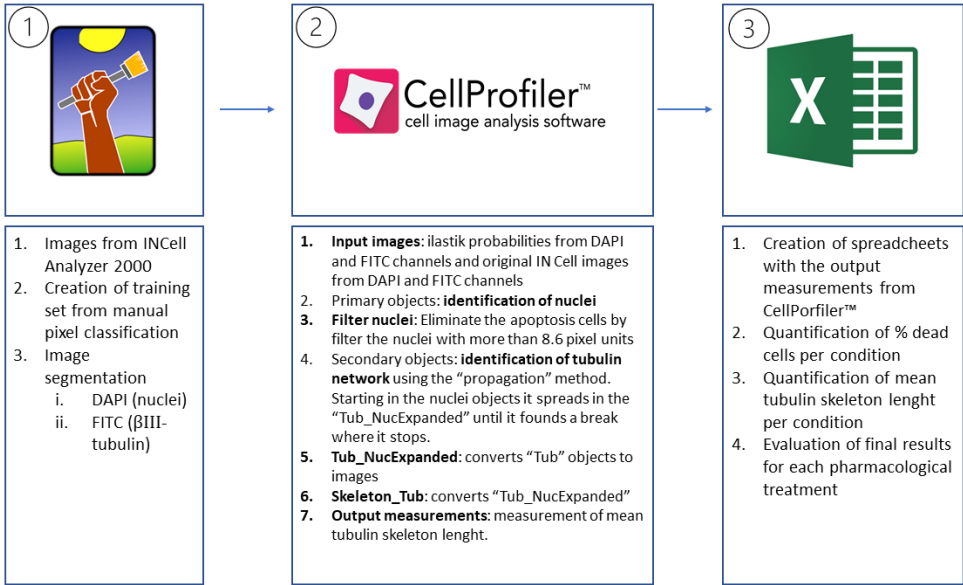


Figure 39. Primary cortical neurons cell model image analysis process.

The procedure developed in ilastik program is similar to the already described for ND7/23 cell line model (Chapter IV, section 2.1.3.). The difference here relied on the use of β III tubulin images (FITC channel in IN Cell Analyzer 2000) instead of α -acetylated tubulin ones. As stated before, this tubulin is less prone to destabilization, allowing a MT-destabilization proper quantification. After ilastik probabilities images creation, the algorithm in CellProfiler™ first

identifies nuclei and filters smaller ones (less than 8.6-pixel units), corresponding to the apoptotic cells. This assumption was based on the work validated and developed by Anilkumar *et al.* [222] that distinguish live and apoptotic neurons by their nucleus characteristic, translating smaller and denser nuclei as apoptotic cells. Afterwards, tubulin objects were identified by the propagation of the live nuclei, assuring therefore that only tubulin fragments derived from the cells were analysed. This was important to eliminate possible background small fragments and also to discern discontinuous skeleton (destabilization effect) from long and continuous skeleton (stabilizing effect). Fig. 40 shows the differences after DMSO and Taxol in the intermediate skeleton outputs.

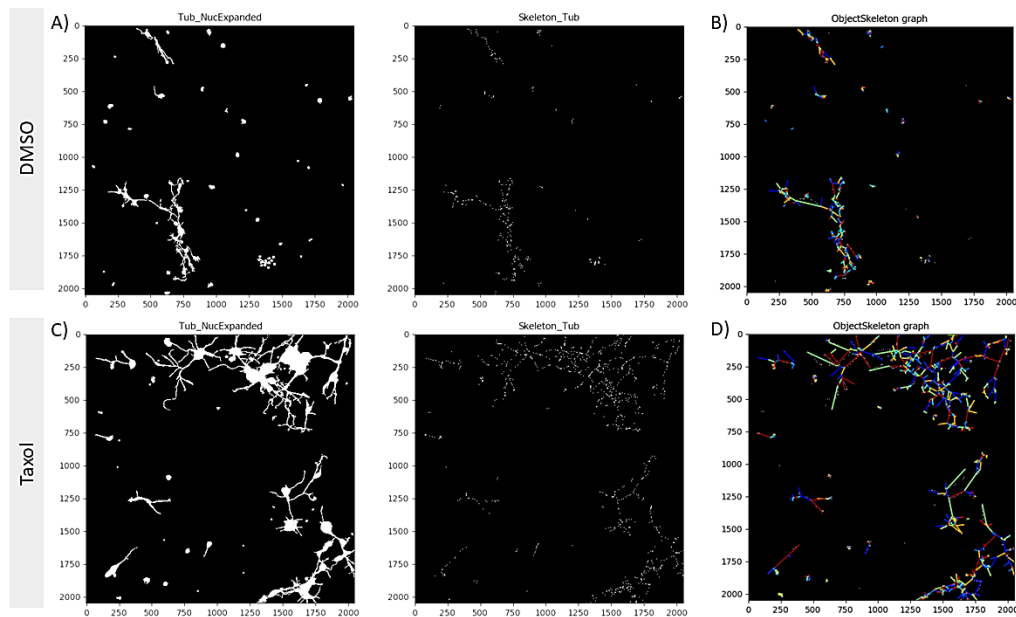


Figure 40. Representative images of outputs from “Skeleton” pipeline in CellProfiler™ software showing the differences regarding DMSO and taxol treatment in the skeleton. (A) Secondary object “Tub_Nuc Expanded” and the image “Skeleton_Tub” created from it in the DMSO condition. (B) Object skeleton graph where are represented trunks (in red), branches (in green), and endpoints (in blue) of neuron skeleton after DMSO treatment. (C) Secondary object “Tub_Nuc Expanded” and the image “Skeleton_Tub” created from it in the Taxol condition. (D) Object skeleton graph where are represented trunks (in red), branches (in green), and endpoints (in blue) of neuron skeleton after taxol treatment. Units are in pixel.

Generally, for both cell seeding densities, the quantifications of average skeleton length correlated with the expected effects for each condition (Fig. 41 (A, C)). After Taxol treatment cortical neurons displayed a bigger average skeleton length than after DMSO treatment and the destabilization effect caused by the Nocodazole agent was translated into a smaller average skeleton length. High standard deviations were reported after all the drug-control treatment, which was in part a signal of the typical heterogeneity observed in primary cell cultures. Regarding the analysis of the percentage of apoptotic cells (Figure 41 (B, D)), although the results show high percentages of neuronal death, this was observed for all pharmacological treatments indicating that the treatments *per se* did not induce major neuronal damage.

Moreover, 26.300 and 39.500 cells/cm² densities revealed suitable for MT remodelling evaluation, since the results after drug-controls treatment were very similar among the conditions. Nevertheless, it was distinguished a different behaviour regarding cortical neurons interconnection. Whereas in 26.300 cells/cm² seeding density the cells were individual, in

39.500 cells/cm² the neurons form a network. Given the higher complexity demonstrated in the seeding density of 39.500 cells/cm², which seems to better represent the *in vivo* cortical neurons behaviour, this was the established seeding density for further experiments.

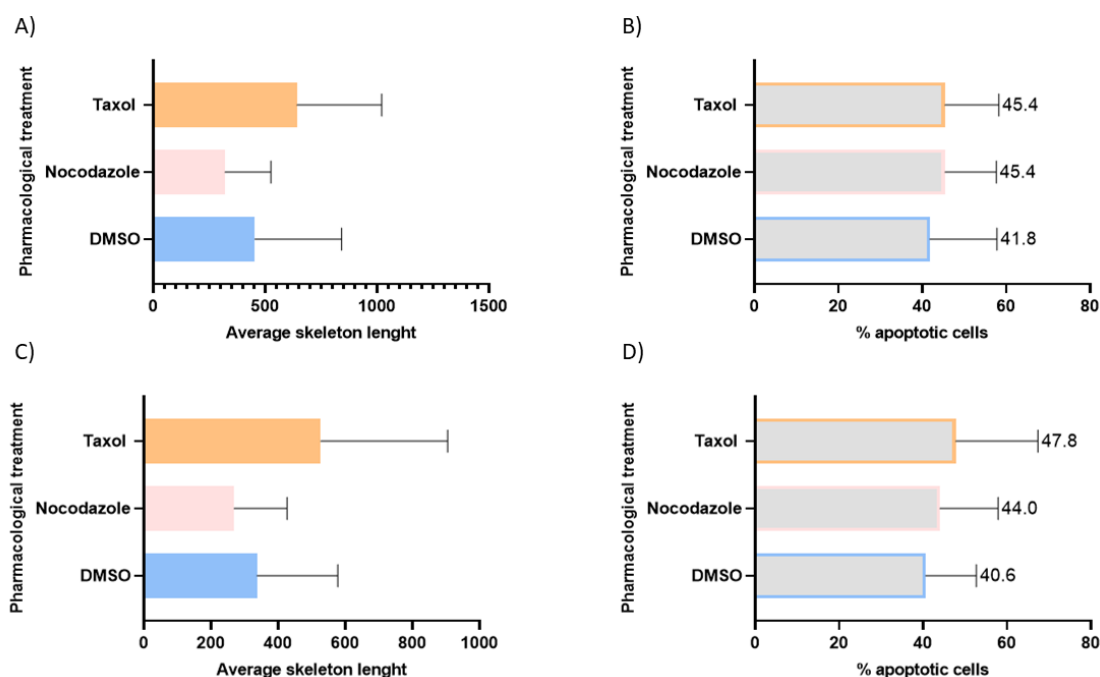


Figure 41. Image analysis quantification results displaying average skeleton length and % apoptotic cells after 4h drug-control treatments of 1.0% DMSO (blue bars), 1 μ M Nocodazole (pink bars), and 100nM Taxol (orange bars). (A) 26.300 cells/cm² primary cortical neurons seeding average skeleton length for each pharmacological treatment; (B) % apoptotic cells in 26.300 cells/cm² primary cortical neurons seeding for each condition; (C) 39.500 cells/cm² primary cortical neurons seeding average skeleton length for each pharmacological treatment; (D) % apoptotic cells in 39.500 cells/cm² primary cortical neurons seeding for each condition. n=3 independent experiments, 2 replicates per condition and 49 fields per replicate. Graphs present Mean $\pm$ SD.

Overall, it was possible to conclude that resorting to the cell morphology evaluation with analysis of α -acetylated and β III tubulins and to the “tubulin skeleton” method it was established the adequate tools for evaluation of candidates to microtubule-stabilization agents in neuronal primary cultures.

2.2.2. Evaluation of positive candidates for microtubule-stabilization in primary cortical neurons model

Independently from the results obtained in the studies performed in ND7/23 cells, all the five compounds (Chapter IV, section 2.1.4.) were also evaluated in the established mouse primary cortical neurons model. This decision was based considering that cell lines might present distinct behaviours from primary cells. Additionally, the two cell models established represent two types of different neurons: i) ND7/23 are immortalized DRGs, located mainly in the PNS; and ii) cortical neurons, part of the CNS. The interconnection between CNS and PNS is represented in some neurodegenerative disorders that end up affecting both systems, therefore it was important to evaluate neuronal cells for both systems.

Each compound was incubated with the primary cortical neurons (39.500 cells/cm² cell density) at a concentration of 100nM, the same tested in section 2.1.3. Taken into consideration the

higher stabilization effects observed in *U. maydis* model the pharmacological treatment was performed at 3 timepoints: 4h, 2h, and 1h, as analysed in the ND7/23 cell line model. The impact of the selected positive candidates on the MT stability was investigated following the tools established in section 2.2.1.

Aprepitant

Regarding Aprepitant effect on primary neurons, firstly after 1h of incubation, it was possible to detect high intensity β III tubulin staining near the nucleus of some neurons, feature not observed after DMSO treatment (Fig. 42). Interestingly, other surrounding cells also showed signs of cytoskeleton degradation with the presence of WD phenotype, intense dots' over β III tubulin network. These observations were confirmed by the average skeleton length (Fig. 43). Although the average skeleton length after 1h treatment was the highest of the 3 time points analysed, this was still lower than DMSO control and similar to Nocodazole quantifications, perhaps a sign of Aprepitant toxic concentration for the cells, also observed in the ND7/23 cells model. Nevertheless, α -acetylated tubulin network was intact after 1h Aprepitant treatment.

At the 2h time point, the Aprepitant agent caused the loss of the β III tubulin network and the destabilization process starts to be visualized in the α -acetylated tubulin, that begins to form retraction bulb-like forms (highlighted by the white arrow in Fig. 42). Ultimately, after 4h treatment with Aprepitant the β III tubulin network of primary cortical neurons remained similar to what was observed at 2h time point and the α -acetylated tubulin network was dotted due to WD (ROI Fig. 42), losing their stability.

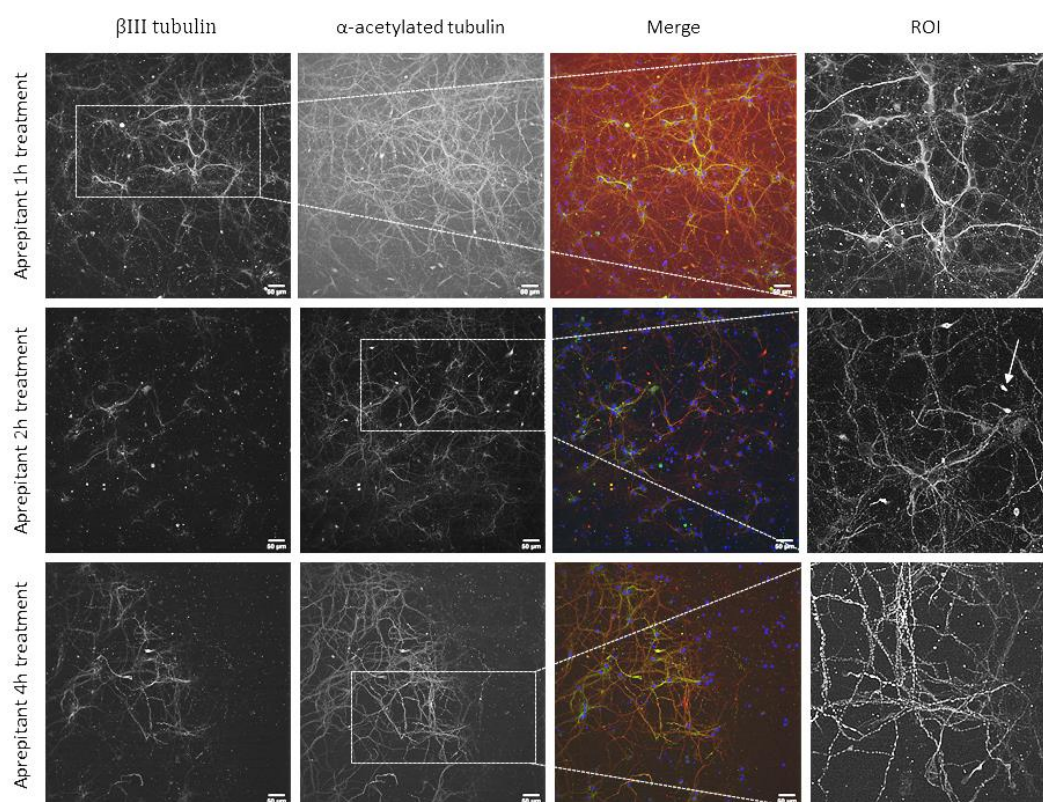


Figure 42. Representative images of acquired channels in IN Cell Analyzer 2000 of primary cortical neurons model at 7 DIV and after 1h, 2h, and 4h 100nM Aprepitant treatment. White arrow highlight retraction bulb-like form. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

The average skeleton length measure in the 2h and 4h treatment conditions (Fig. 43) was lower than 1h time point, corroborating cell morphology qualitative analysis. Overall, the Aprepitant compound revealed a mechanism of MT network disruption. However, considering the certain level of stabilization observed in some neurons after 1h Aprepitant treatment it can be considered that the concentration used was higher than the required for MT stabilization, causing the loss of network. One important note regarding Aprepitant treatment in the primary cortical neurons model is the percentage of apoptotic cells (Fig. 43 (B)) which was on the same range of the control-drugs treatment, allowing the conclusion that Aprepitant does not seem to be toxic for the primary cortical neurons.

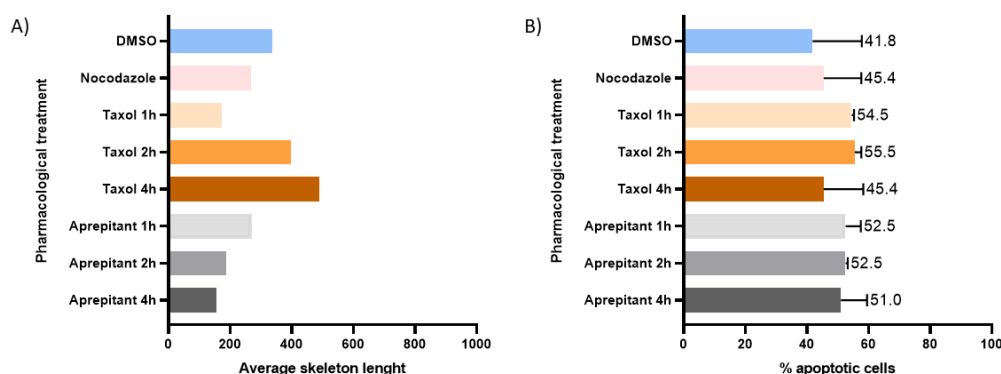


Figure 43. Image analysis quantification results displaying average skeleton length and % apoptotic cells after 4h drug-control treatments of 1.0% DMSO and 1 μ M Nocodazole and 1h, 2h, and 4h treatments of 100nM Taxol and 100nM Aprepitant. (A) Average skeleton length for each condition; (B) % apoptotic cells for each condition. For all graphs, blue bars refer to DMSO, pink bars refer to Nocodazole, orange bars refer to Taxol and grey bars refer to Aprepitant (lighter the colour, lower the time point for all conditions). n=1 independent experiment, 2 replicates per condition and 49 fields per replicate. Graphs present Mean $\pm$ SD.

Atracurium besylate

Concerning the Atracurium besylate effect on primary neurons, firstly after 1h treatment it was visible the hipper stabilization of the β III tubulin represented by strong labelling intensity (Fig. 44) and thicker cytoskeleton around the nuclei in cells. Accordingly, the stabilization effect also had impact in the α -acetylated tubulin where a MT network denser than after DMSO and Taxol treatment was observed (Fig. 35). The microtubule-stability effect of Atracurium besylate after 1h treatment was corroborated by the quantification of the MT network with an average skeleton length considerably larger than with DMSO and Taxol (Fig. 45 (A)). Nonetheless, the MT-stability demonstrated after 1h of treatment with Atracurium besylate was decreasing for higher time points. After 2h of treatment, the α -acetylated tubulin network was not as dense and started to be similar to what was observed after DMSO treatment (Fig. 35). Furthermore, the β III tubulin starts to demonstrate signs of destabilization, such as the presence of dots instead of a continuous tubulin filament (Fig. 44). For the longest incubation time, the β III tubulin was almost disintegrated, and the α -acetylated tubulin begin to destabilize as well, with loss of the network and the appearance of fragments of tubulin.

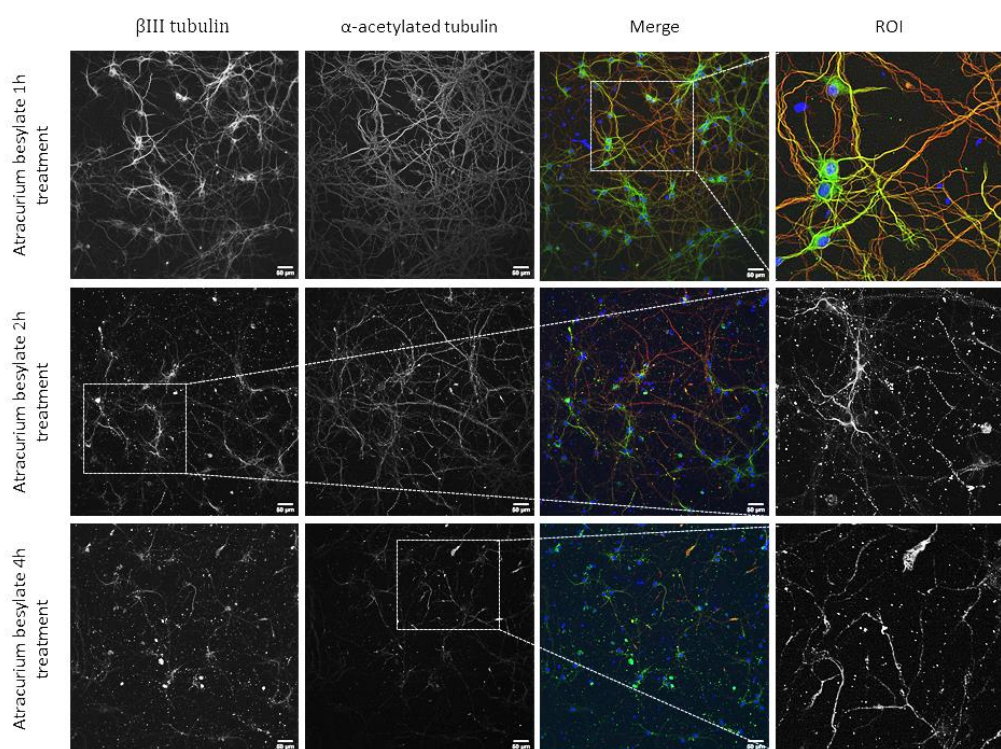


Figure 44. Representative images of acquired channels in IN Cell Analyzer 2000 of primary cortical neurons model at 7 DIV and after 1h, 2h, and 4h 100nM Atracurium besylate treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

As expected after cell morphology analysis, 2h and 4h treatment with Atracurium besylate reveal a microtubule-destabilization mechanism by presenting average skeleton lengths similar to Nocodazole MT network quantifications (Fig. 45 (A)). Nevertheless, Atracurium besylate does not show signs of causing neuron toxicity at the evaluated time points since percentage of apoptotic cells after the treatment were in the 52.0-53.0%, similar to Taxol treatment levels.

Atracurium besylate has an undeniable microtubule-stabilizing effect on primary cortical neurons. The preliminary analysis showed an excess of MT stability for 2h and 4h time points, indicating that more studies should be conducted in order to optimize the therapeutic effect.

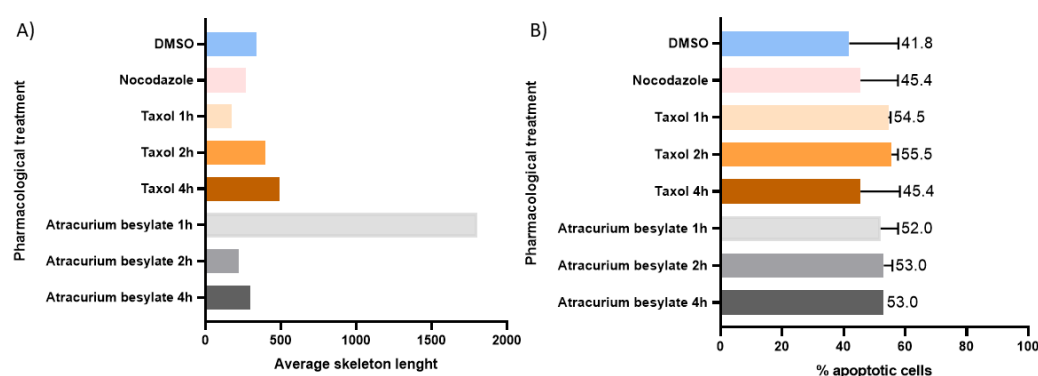


Figure 45. Image analysis quantification results displaying average skeleton length and % apoptotic cells after 4h drug-control treatments of 1.0% DMSO and 1 μ M Nocodazole and 1h, 2h, and 4h treatments of 100nM Taxol and 100nM Atracurium besylate. (A) Average skeleton length for each condition; (B) % apoptotic cells for each condition. For all graphs, blue bars refer to DMSO, pink bars refer to Nocodazole, orange bars refer to Taxol and grey bars refer to

Atracurium besylate (lighter the colour, lower the time point for all conditions). n=1 independent experiment, 2 replicates per condition and 49 fields per replicate. Graphs present Mean $\pm$ SD.

Danazol

The third compound to be evaluated in the primary cortical neurons model was Danazol. After 1h drug treatment neurons demonstrated a well-developed network composed by α -acetylated tubulin. However, the β III tubulin began to destabilize, demonstrated by the presence of dots instead of a continuous tubulin filament (Fig. 46). The signs of MT-destabilization continued after 2 and 4h of treatment. At the 2h time point a discontinuous network in the α -acetylated tubulin was observed. Finally, the completely lost of all tubulin network was achieved around the 4h time point (Fig. 46).

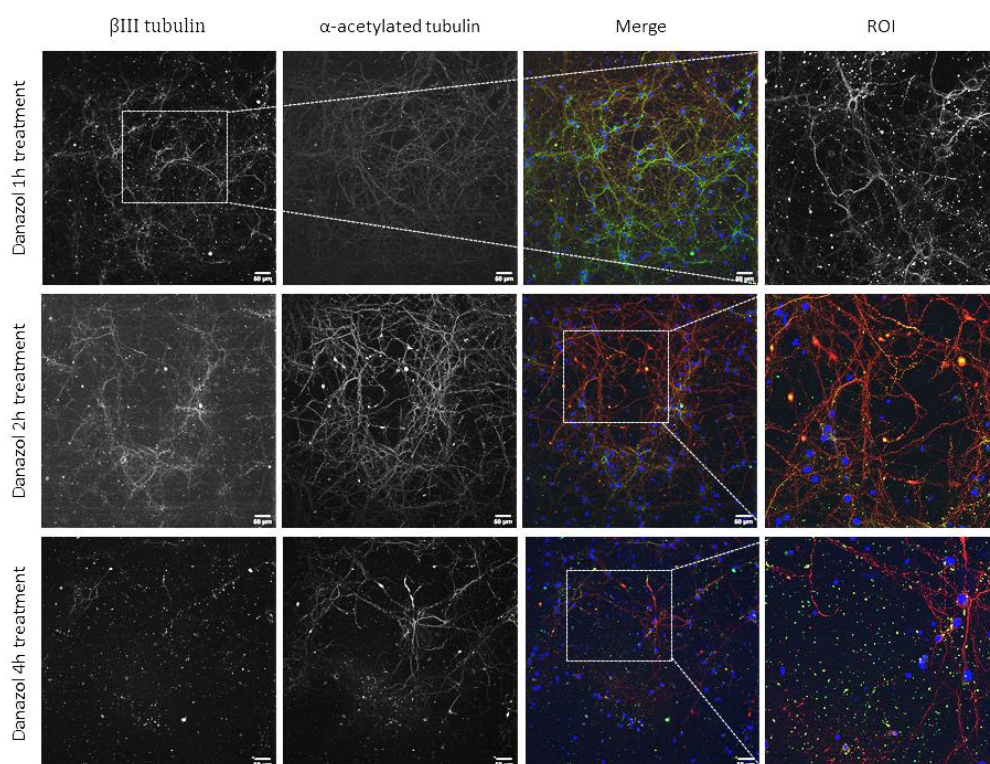


Figure 46. Representative images of acquired channels in IN Cell Analyzer 2000 of primary cortical neurons model at 7 DIV and after 1h, 2h, and 4h 100nM Danazol treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

Contrary to what was demonstrated in the *U. maydis* and ND7/23 cells models where Danazol demonstrate a certain capacity for microtubule-stability, the evaluation in the primary cortical neuron model caused destruction of the tubulin network, as shown in Fig. 47 (A). The average skeleton length was considerably smaller than the DMSO condition and even inferior to the Nocodazole treatment. Overall, one would think that Danazol would be toxic to the cortical neurons, nonetheless the percentage of apoptotic cells after drug treatment revealed the same levels as the demonstrated for the controls (Fig. 47 (B)).

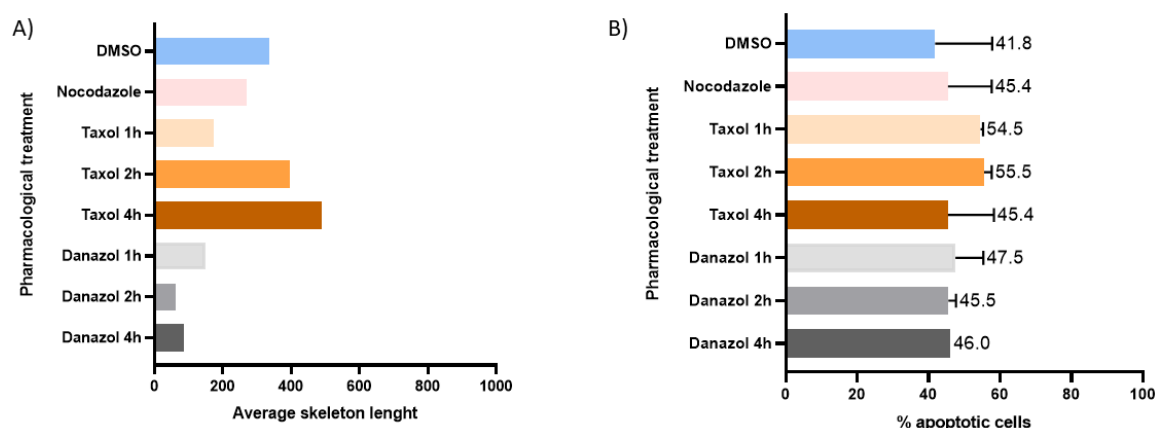


Figure 47. Image analysis quantification results displaying average skeleton length and % apoptotic cells after 4h drug-control treatments of 1.0% DMSO and 1 μ M Nocodazole and 1h, 2h, and 4h treatments of 100nM Taxol and 100nM Danazol. (A) Average skeleton length for each condition; (B) % apoptotic cells for each condition. For all graphs, blue bars refer to DMSO, pink bars refer to Nocodazole, orange bars refer to Taxol and grey bars refer to Danazol (lighter the colour, lower the time point for all conditions). n=1 independent experiment, 2 replicates per condition and 49 fields per replicate. Graphs present Mean $\pm$ SD.

Disulfiram

Regarding Disulfiram effect on primary cortical neurons, firstly after 1h of treatment it was visible β III tubulin lost. However, a few cells still had signs of β III tubulin stabilized, with intense staining of this tubulin type near the cellular body (Fig. 48), which might indicate that the destabilization of the tubulin network could be caused by an excess of microtubule-stabilization effect. Nonetheless, the α -acetylated tubulin network was similar to the observed with the DMSO control. The average skeleton length revealed numbers similar to Nocodazole treatment (Fig. 49 (A)), which can in part be justified by the balance between neurites still showing some β III tubulin stabilization and others where the network was lost.

After β III tubulin loss, at the 2h time point, also α -acetylated tubulin started to disintegrate presenting WD phenotype. While after 4h drug treatment were also present retraction bulb-like structures (Fig. 48), characteristic of microtubule-destabilization. This mechanism was confirmed with the quantification of the average skeleton length shown in Fig. 49 (A), where measurements for 2h and 4h conditions were even lower than the average skeleton length after 1h Disulfiram and Nocodazole treatment. Surprisingly, the percentage of apoptotic cells after 1 and 4h treatment was on average 61.5% and 58.0%, respectively, higher numbers than the controls.

In general, Disulfiram showed a microtubule-stabilizing effect on primary cortical neurons. The pilot experiments indicate the preliminary excess of MT stability for all the time points. To confirm its capacity on MT modulation, further studies should be conducted.

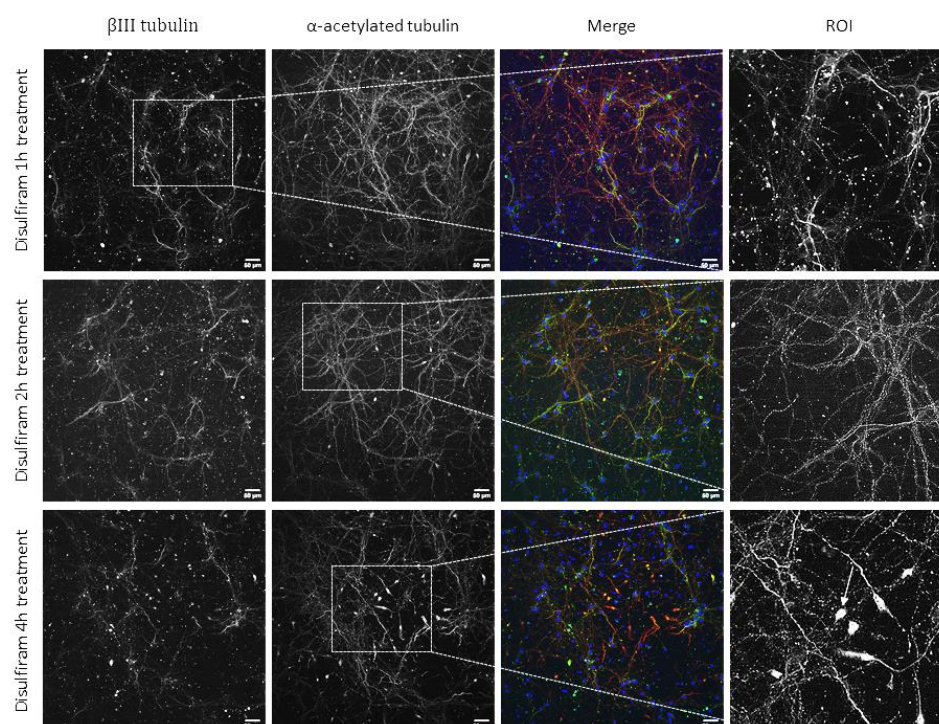


Figure 48. Representative images of acquired channels in IN Cell Analyzer 2000 of primary cortical neurons model at 7 DIV and after 1h, 2h, and 4h 100nM Disulfiram treatment. White arrows highlight retraction bulb-like structures. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

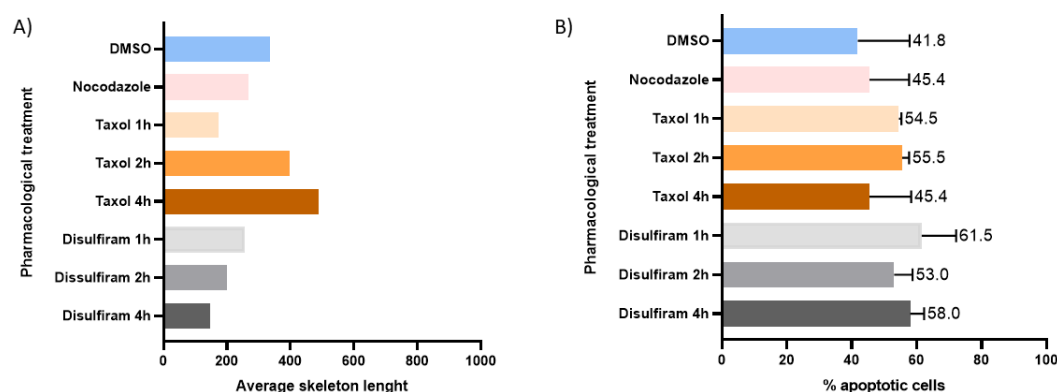


Figure 49. Image analysis quantification results displaying average skeleton length and % apoptotic cells after 4h drug-control treatments of 1.0% DMSO and 1 μ M Nocodazole and 1h, 2h, and 4h treatments of 100nM Taxol and 100nM Disulfiram. (A) Average skeleton length for each condition; (B) % apoptotic cells for each condition. For all graphs, blue bars refer to DMSO, pink bars refer to Nocodazole, orange bars refer to Taxol and grey bars refer to Disulfiram (lighter the colour, lower the time point for all conditions). n=1 independent experiment, 2 replicates per condition and 49 fields per replicate. Graphs present Mean $\pm$ SD.

Pranlukast

Finally, it was evaluated the microtubule-stability capacity of Pranlukast in the primary cortical neuronal model. After 1h drug treatment an intact and dense α -acetylated and β III tubulin network was demonstrated (Fig. 50). Interestingly, thicker axons were also shown, and the signs of MT-stability were evident. However, the effect was similar to the Taxol treatment and did not seem as strong as the observed after 1h Atracurium besylate treatment. The average skeleton

length for 1h time point (Fig. 51 (A)) was higher than the DMSO control and 1h Taxol treatment, endorsing the idea of Pranlukast has a microtubule-stabilization agent.

After 2h of Pranlukast treatment, the MT-stabilization effect previously observed begin to be in excess for the mouse primary cortical neurons. Thus, inverting the tubulin dynamics from stabilization to destabilization with α -acetylated tubulin demonstrating retraction bulb-like structures (Fig. 50). Accordingly with these alteration in MT modelling, β III tubulin after 2h treatment was completely lost in some cells and still present with considerable intensity in others, as shown in ROI of Fig. 50.

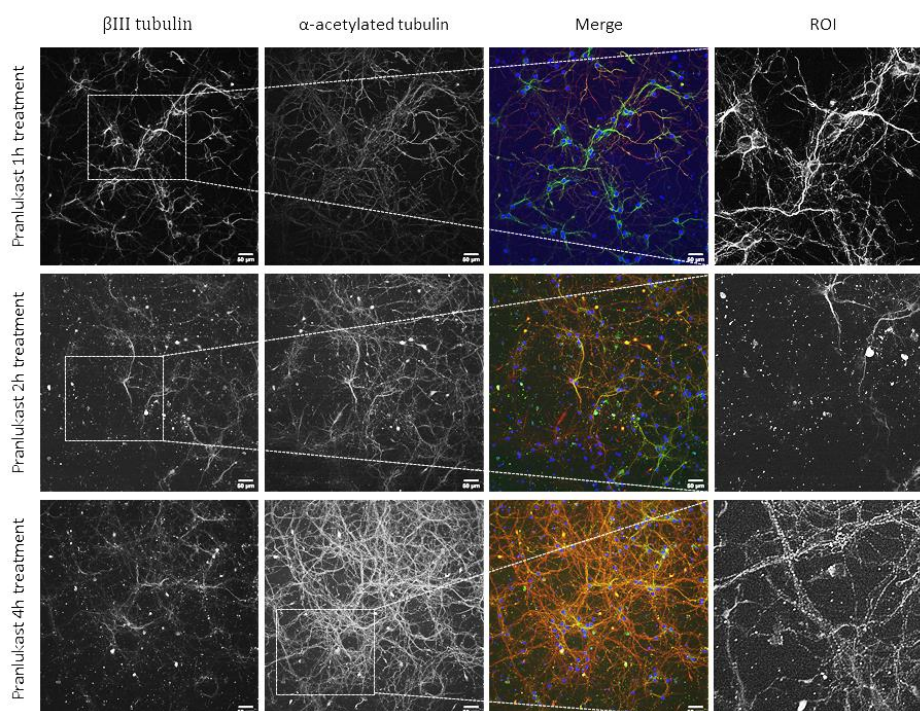


Figure 50. Representative images of acquired channels in IN Cell Analyzer 2000 of primary cortical neurons model at 7 DIV and after 1h, 2h, and 4h 100nM Pranlukast treatment. β III tubulin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

The average skeleton length after 2h Pranlukast treatment was lower than the measured after 4h time point (Fig. 51 (B)). These results are contradictory to the morphological analysis where MT-destabilization was observed after 4h treatment. Despite having increased as incubation times rise, the percentage of apoptotic cells remained inside the range of apoptotic cells quantified after drug-controls (Fig. 51 (B)).

Overall, it was possible to consider that Pranlukast has a microtubule-stabilizing effect on primary cortical neurons. The preliminary analysis showed an excess of MT stability for higher time points. These data will be confirmed with further studies on different time points and concentration conditions.

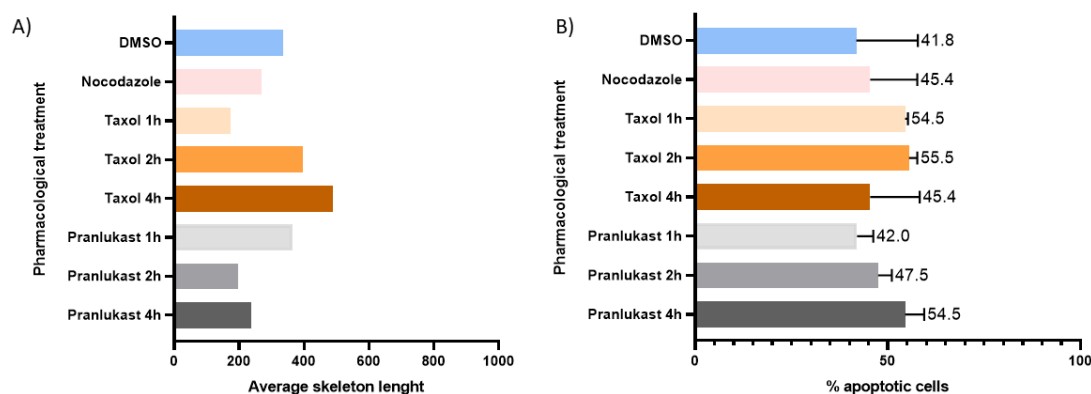


Figure 51. Image analysis quantification results displaying average skeleton length and % apoptotic cells after 4h drug-control treatments of 1.0% DMSO and 1 μ M Nocodazole and 1h, 2h, and 4h treatments of 100nM Taxol and 100nM Pranlukast. (A) Average skeleton length for each condition; (B) % apoptotic cells for each condition. For all graphs, blue bars refer to DMSO, pink bars refer to Nocodazole, orange bars refer to Taxol and grey bars refer to Pranlukast (lighter the colour, lower the time point for all conditions). n=1 independent experiment, 2 replicates per condition and 49 fields per replicate. Graphs present Mean $\pm$ SD.

For each treatment and each time point were analysed 2 replicas and 49 frames per replica and this alteration between frames with complete loss of MT network and frames with stabilized tubulin was detected all over the 98 frames analysed. The MT network formed by the primary cortical neurons is not equally dense throughout the well at pre-treatment stage, there are variations between individual neurons and, consequently, their response to the surrounding environment. These variations in distribution result in differences in MTs expression and in the network structure. So, it is relevant to consider these different morphologies when analysing the drug's effects on the MT dynamic because it is expected that cortical neurons around a denser skeleton network are more resistant to drug effects whether it is stabilization or destabilization of tubulin [213]. The skeleton length quantification method focusses on important aspects of the cells MTs network, but since it gives the average quantification it balances the 2 situations reported. Thus, the average skeleton length method translates up to a point the globally of MTs stabilization/destabilization but does not always translate the two variations individually.

Considering the skeleton length quantification method for evaluation of MT remodelling this issue are not be notice because the average will balance the 2 situations reported and, therefore, in this case no conclusions can be taken.

2.3. Microfluidics: one step closer to a complex *in vitro* model

2.3.1. Microfluidic device characterization

PDMS microfluidic devices were produced through a template and all conditions were replicated in every experiment. To ensure that the fabrication process was successful, and microfluidics had the expected pattern and dimensions, samples were analysed using SEM (Fig. 52).

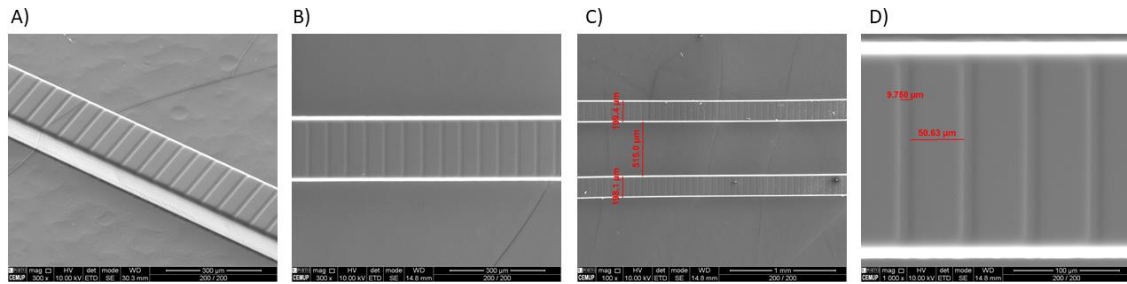


Figure 52. Representative SEM images of PDMS microfluidic devices. (A) Side view image of the centre of the microfluidic device. (B-D) Upper view images of the microfluidic device. (C-D) Images of the microgrooves incorporated in the microfluidic with measurements of channels and microgrooves and distance between channels. Present values correspond to measurements made during acquisition of the SEM images, n=1 independent experiment. All measurements are in μm . Scale bars: (A-B) 300 μm ; (C) 1mm; (D) 100 μm .

The SEM images of the microfluidic device showed a regular surface, indicating a successful production. To acquire a general visualization of the center channels and their microgrooves it was used a side view and an upper view to ensure their alignment and to measure the corresponding distances (Fig. 52). The central channels had a width of approximately 200.00 μm (with a width of 199.4 μm in one channel and 198.1 μm in the other) and distanced between them is 515.0 μm . Each microgroove had a width of 9.750 μm and the space between the two of them is 50.63 μm .

Microfluidic device presented the expected dimensions and distances of the channels and microgrooves. The similarity between theoretical and real values was crucial because the template was designed for the production of microfluidic devices with specific microgrooves patterns and dimensions to allow neurite passage across the device through the microgrooves preventing the cell bodies from entering.

2.3.2. Optimization of cell density in microfluidic platform

The microfluidic devices was produced to create a cell culture model where neuronal cells were grown in an environment that more closely mimic the *in vivo* conditions by physically isolate neuronal cell bodies from developing neurites. In order to ensure correct primary cortical neurons growth and physical separation throughout the microgrooves, various cell seeding densities were tested in the microfluidic devices.

Prior to cell culture, the microfluidic devices were sterilized and pressed against a PDL-coated coverslip (to allow microscopy analysis), ensuring a correct bounding to avoid medium leakage and air bubbles during the seeding process. Mouse primary cortical neurons were cultured in the microfluidics in 3 different high densities, 500.000 cells, 800.000 cells and 100.000 cells.

At 7 DIV, the cells were fixed and stained for β III tubulin and actin (by staining phalloidin) and observed by confocal microscope (Fig. 53).

For a seeding density of 500.000 cells/microfluidic, primary cortical neurons formed a dense network in the soma channel of the microfluidic device (Fig. 53 (A)). It was also possible to see that this lower cell density was sufficient for the growth of projections of cortical neurons from the soma channel through the microgrooves to the axonal channel. Although the complete extension of neurites to the other channel was not yet achieved completely in the entire length

of the channel, the neurites seem to be present in the majority of the microgrooves creating a physical separation from soma and neurites.

On other hand, a cell density of 800.000 cells/microfluidic allowed the growth of a strong network between primary cortical neurons in the central channel, with neurites passing to the other side channel in the majority of the microgrooves (Fig. 53 (B)).

Finally, in the microfluidic with the highest seeding density of 1.000.000 cells/microfluidic was not observed the growth of primary cortical neurons evenly in the central channel (Fig. 53 (C)). This phenomenon could be caused by an excess of cells due to a higher cell density than the required, causing some cell death and growth of a less number of cells. Nevertheless, in the areas where there was growth of primary cortical neurons their neurites were able of passing throughout the microgrooves creating a physical separation from soma and neurites.

Accordingly, with the confocal microscopy images, all the seeding densities evaluated were enough for the proper growth of primary cortical neurons throughout the microgrooves, presenting similar conditions for the physical separation of soma and neurites. Based on these observations was selected the lowest seeding density (500.000 cells/microfluidic) to proceed with the establishment of a microfluidic model as a tool for evaluation of microtubule-stabilizing compounds.

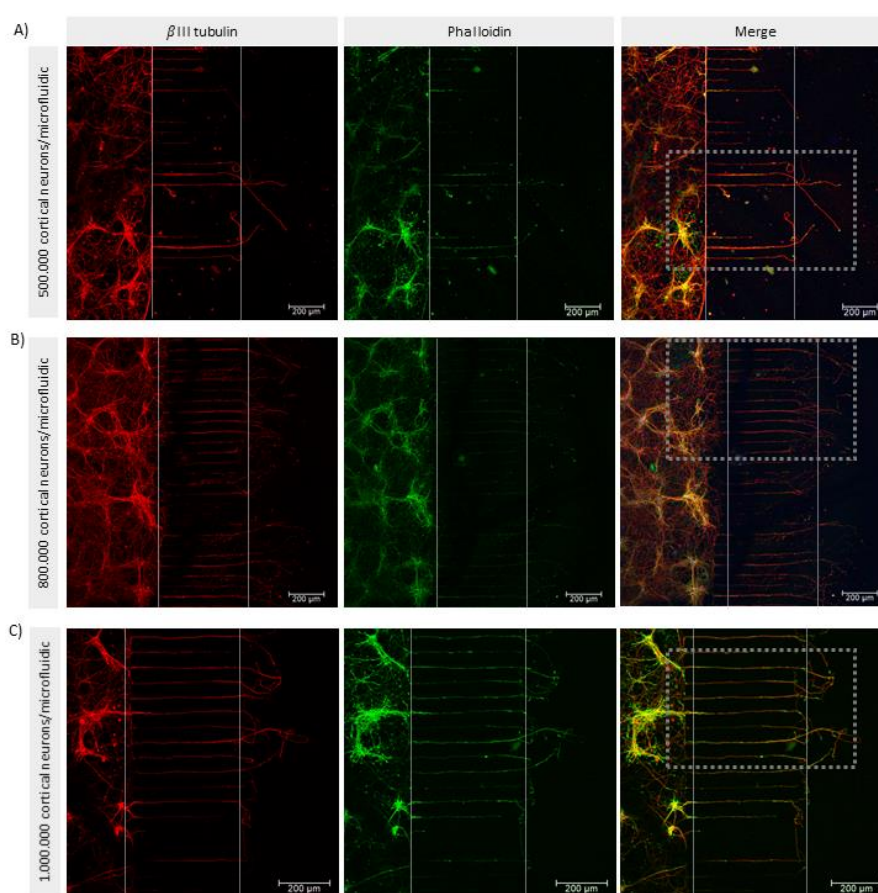


Figure 53. Representative images of acquired channels in confocal microscope of primary cortical neurons cultured in microfluidic device at 7 DIV. (A) Cell density of 500.000/microfluidic. (B) Cell density of 800.000/microfluidic. (C) Cell density of 100.000/ microfluidic. Phalloidin (green), β III tubulin (red) counterstained with Hoechst (blue). Scale bars: 200 μ m.

2.3.3. Establishment of drug-controls for microtubule-stabilizing agents in microfluidic devices: DMSO, Nocodazole and Taxol

For the establishment of the microfluidic model as a tool in the evaluation of potential microtubule-stability agents the drug-controls effects were analysed in primary cortical neurons cultured in the PDMS device. The cells were treated with 1.0% DMSO, 1 μ M Nocodazole and 100nM Taxol for 2 days [223]. After, the devices were fixed and stained for α -acetylated tubulin and actin (by staining phalloidin) and observed by confocal microscope.

In the 2D well-plates where the primary cortical neurons model were cultured all neurons structure are in the same environment. Contrary, in the microfluidic devices, the physical separation of cell bodies and neurites allowed by the microgrooves enables a pharmacological treatment targeting separately the axons. Therefore, the evaluated compounds were added to one of the inlets linked to the axonal channel.

After DMSO treatment (Fig. 54 (A)) the neurites behaviour crossing the microgrooves maintained similar to the observed without treatment for the same cell density (Fig. 53 (A)) and a normal α -acetylated tubulin network was formed in the soma channel. However, the phalloidin staining was not equally distributed in the channel and no actin extensions were detected throughout the microgrooves, as shown in Fig. 54 (A). Since both Nocodazole and Taxol are solubilized in DMSO and the shortage of actin filaments was not detected in these controls it is likely that this was not caused by DMSO toxicity but might be associated with culture diversities. To confirm the non-toxicity of DMSO in our microfluidic model further studies should be performed to confirm the integrity of actin filaments, one of the key pieces for the cytoskeleton together with tubulin.

Regarding the negative control, the treatment with the microtubule-destabilizer agent Nocodazole is displayed in Fig. 54 (B) where it was possible to detect the complete absence of neurites extended in the microgrooves without not only actin but also α -acetylated tubulin. In the soma channel it was still visible a MT network, which indicates that the absence of extensions were due to the MT-destabilization induced by the Nocodazole treatment. Curiously, it was possible to conclude that the antimitotic effect of Nocodazole spread throughout the axons and reached the neuronal cell body in the soma channel opposite to the treatment, as highlighted by the ROI in Fig. 54 (B). It was observed destabilization of α -acetylated tubulin together with shrinkage in actin filaments on the areas closest to the microgrooves.

Finally, the positive control of the microtubule-stabilization agent Taxol was established in the microfluidic model, as shown in Fig. 54 (C). Comparing with the axons extensions after DMSO treatment, it was possible to observe a MT-stabilization effect since neurites are undoubtedly thicker and with higher staining. Regarding the capacity of Taxol in reaching the neuronal soma, no conclusions can be withdrawn since although the MT network inside the channel seems more complex than the formed after DMSO treatment, the differences must be quantified and the cell density pre- and after-treatments accessed.

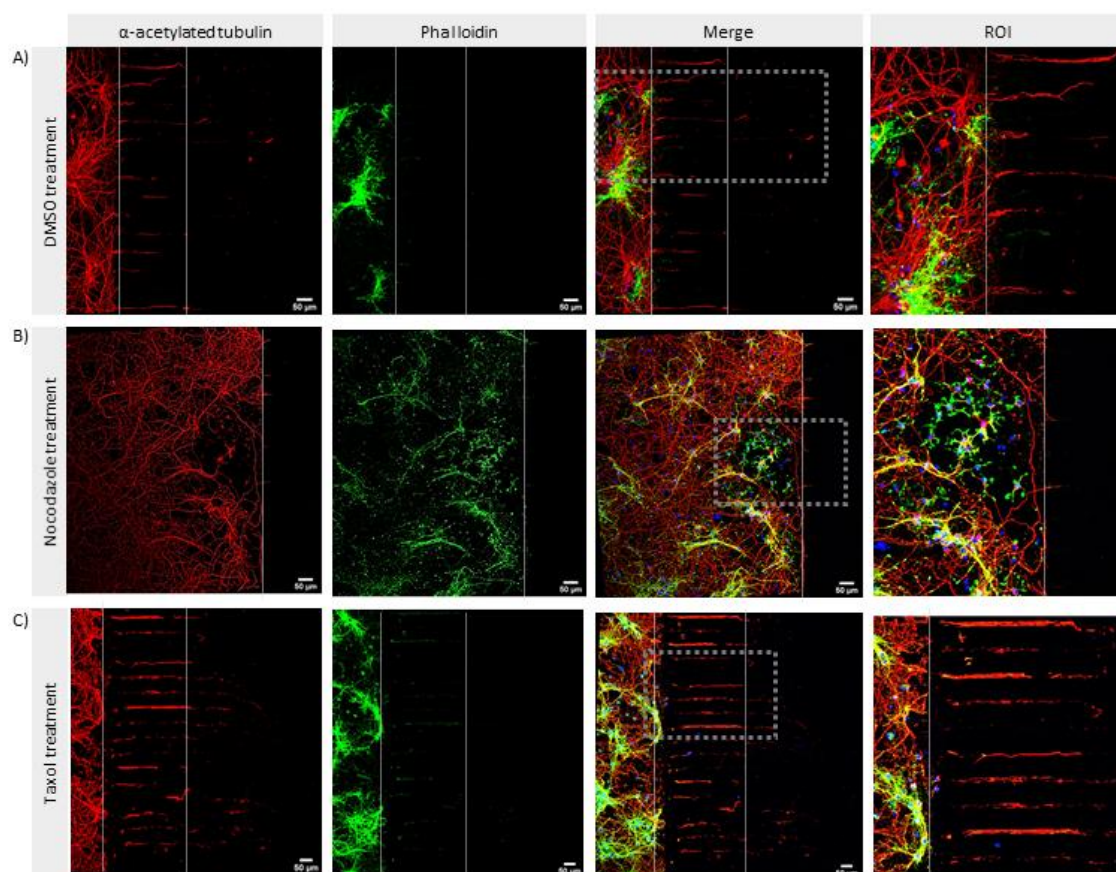


Figure 54. Representative images of acquired channels in confocal microscope of primary cortical neurons cultured in microfluidic device at 7 DIV after 2 days of control-drug treatments. (A) 1.0% DMSO treatment; (B) 1 μ M Nocodazole treatment; (C) 100nM Taxol treatment. Phalloidin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

In spite of some MTs network variations detected, it was possible to establish the microfluidic device has a complex tool for the evaluation of candidates to microtubule-stabilization compounds. This model shows as an opportunity for exposure of MTs neurites to drug separately from neuronal cell bodies in a similar way to what can occur under physiological conditions.

2.3.4. Evaluation of positive candidates for microtubule-stabilization in microfluidic devices

In the microfluidic device model the pharmacological treatments were again evaluated in primary cortical neurons. Nevertheless, the different neurons disposition and the complex environment given by the microfluidic device, a 2.5D model, can display different conclusions regarding microtubule-stability capacities. So, no pre-selection was made and the 5 drugs were analysed in this tool. As it was tested in the other 2 cell models, the selected positive compounds were analysed at the same concentration as Taxol (100nM). However in this tool the treatment was made only at 1 time point, during 2 days as suggested by Gornstein *et al.* for analysis of MT-stability after pharmacological treatment in microfluidic devices [223].

The Aprepitant treatment revealed an interesting effect in MTs of primary cortical neurons cultured in the microfluidic device, as shown in Fig. 55 (C). When evaluated in 2D primary cortical neuronal model Aprepitant compound did not reveal a pronounced microtubule-stability

effect, with the maximum effect reached after 1h treatment that reached an average skeleton length lower than DMSO control. However, some neurons showed MT-stability morphologies. In the microfluidic model Aprepitant could induce MT-stability, the neurites developed through the microgrooves were in higher number than DMSO and Taxol conditions and showed a greater extensions. Almost all the neurites were elongated until the axon channel. Regarding the cytoskeleton network developed in the feeding channel it was similar to the observed after DMSO treatment. However, to assure the absense of drug effect in the soma channel the cells network must be quantified pre- and after-treatment. In case of confirmation of similar networks pre- and after Aprepitant treatment it might be an indion that the stabilization effect had not reach the neurons soma and Aprepitant only hit in the physical separated neurites on the axon channel.

The second candidate to be evaluated was Atracurium besylate. The neurites at the microfluidic device revealed an MT hyperstabilization effect induced by these compound (Fig. 55 (D)). In accordance with the results after Atracurium besylate treatments in primary cortical neurons model where it demonstrate an average skeleton length considerably superior when compared with DMSO and even Taxol. In Fig. 55 (D) one can see that axons extensions were in all the microgrooves passing consistently through them and reaching aplenty into the axon channel. Furthermore, the α -acetylated tubulin staining was more intense than in any other conditions, including the effect caused by Taxol treatment and the MTs were thicker and tenser, signs o microtubule-stability. The same stability is demonstrated by the dense α -acetylated tubulin network formed in the channel where the neuronal soma were located, indicating that Atracurium besylate microtubule-stability effect reached the neurons cell bodies travelling throught the axons extensions. Furthermore, the presence of actin filaments in the soma channel was a sign of an healthy culture.

The treatment with Danazol in the microfluidic model was in accordance with the conclusions taken from primary cortical neuron model revealing no microtubule-stability effect. As shown in Fig. 55 (E), Danazol did not prove neurotoxic since neurites were passing through microgrooves in the same manner as after DMSO treatment (Fig. 55 (A)). The cytoskeleton network of α -acetylated tubulin and actin filaments showed no morphological disturbances in the soma channel, however it did not exhibit modulation of the MTs at this time point and concentration.

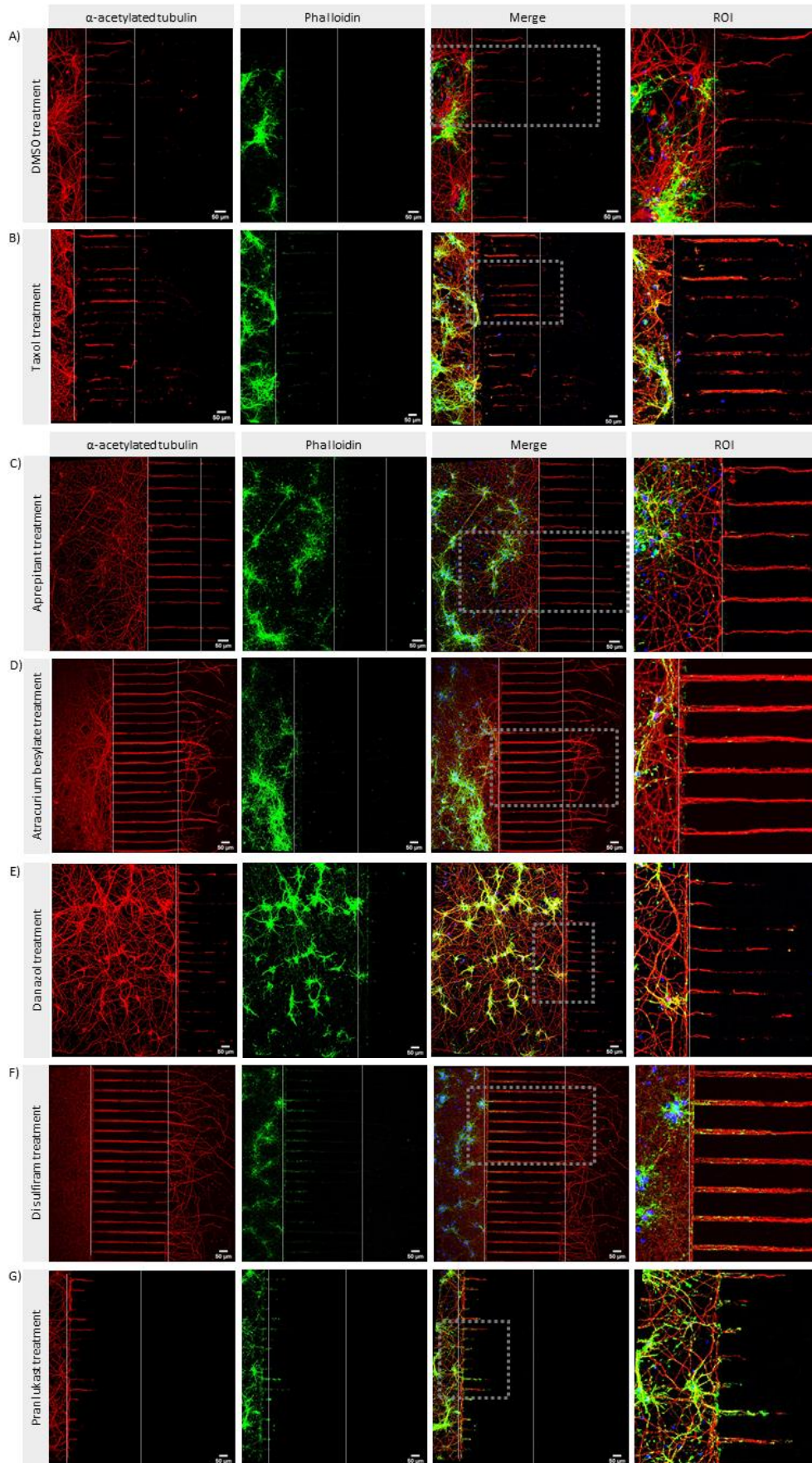


Figure 55. Representative images of acquired channels in confocal microscope of primary cortical neurons cultured in microfluidic device at 7 DIV after 2 days of drug treatments. (A) 1.0% DMSO treatment; (B) 100nM Taxol treatment;

(C) 100nM Aprepitant treatment; (D) 100nM Atracurium besylate treatment; (E) 100nM Danazol treatment; (F) 100nM Disulfiram treatment; (G) 100nM Pranlukast treatment. Phalloidin (green), α -acetylated tubulin (red) counterstained with Hoechst (blue). Scale bars: 50 μ m.

The same way as Atracurium besylate, Disulfiram compound also demonstrated a MT hyperstabilization effect (Fig. 55 (F)), although this outcome was not demonstrated after Disulfiram treatment in primary cortical neuron model. Axon's extensions after Disulfiram treatment were inflexible through the microgroove's length reaching abundantly the axonal channel. The morphology and high number of MTs passing throughout microgrooves was accomplished due to their polar growth towards the Disulfiram compound as a consequence of its MT-stability effect. To notice that contrary to what was observed after the other positive candidates' treatment, with Disulfiram the actin filaments was in a greater amount at the neural cell bodies channel, and it was even detected actin filaments passing through the microgrooves (Fig. 55 (F)).

Finally, it was evaluated in the microfluidic model Pranlukast. In the primary cortical neuron model after 1h treatment Pranlukast revealed a capacity to induce microtubule-stability however, as demonstrated in Fig. 55 (G), the cytoskeleton morphology does not allow to draw conclusions regarding microtubule-stability capacity of Pranlukast in the microfluidic model. The neurites were not elongated through all the microgrooves; indeed the axons extensions were smaller than after DMSO treatment. Despite presenting short tubulin extensions the α -acetylated tubulin was compact and with intense staining and the presence of actin filaments in the edges of the neuronal extensions, could be a sign of an active synapse and MT-stabilization or can be signs of initial cell death caused by MT stability toxicity. For now no conclusions can be withdrawn and further studies should be performed to assess Pranlukast MTs modulation effect.

Conclusion

Neurodegenerative disorders represent a wide group of completely different pathologies. Although neurodegeneration is associated with CNS, the involvement of PNS is relatively common in few proteinopathies of the brain [224]. While AD and ALS are CNS pathologies, hereditary sensory and autonomic neuropathies (HSANs) are associated with PNS dysfunction. However, the interconnection between CNS and PNS is represented in some neurodegenerative disorders end up affecting both systems. PD is usually commonly thought of as a disorder of the brain (CNS) that spreads to the PNS, but Borghammer, P. *et al.* suggested that its onset is in the PNS, in the nerves around the gut, and only then spreads to the brain [225]. Therefore the neuron models established in these project ND7/23 cell line, primary cortical neurons and microfluidic device cover both CNS and PNS and make the most of the best characteristics of immortalized cell models and primary cell models.

The ND7/23 cells enabled the establishment of a tool where immortalized DRGs, present in the PNS, were studied for effects of microtubule-stabilizing agents. Implementing a cell line model had interesting characteristic for a preliminary evaluation of *U. maydis* HTS screening such as easy scale up, reproducibility and simple manipulation. Differentiation of the ND7/23 cells induces outgrowth of long unbranched neurons creating a useful model for evaluation microtubule-stability agents by analysing cell morphology and collar occupancy quantification.

In the ND7/23 cells model Aprepitant and Disulfiram reveal a MT-stability agent with higher effect for longer time points of 2h and 4h. Atracurium besylate also demonstrated properties of microtubule-stability agent with indications of abrupt and strong MT modulation effect. Since 1h time point there had been no effect, at 2h time point the MT-stabilization was strong and at 4h treatment already showed excess of stability signs inverting to retraction bulb-like structures. Contrarily, Danazol evaluation in the ND7/23 cells model did not allow to withdraw conclusions. Finally, Pranlukast was the selected compound that more consistently reveal MT-stability properties in all the tested time points.

The results in the ND7/23 cell line model should take into consideration that in contrary to the observed after Taxol treatments, some drug candidates demonstrate the ability to induce neuronal branching namely in the tips and these MT alterations were not quantified in the collar occupancy method. Thus, the collar occupancy method is suitable for advanced MT-stability levels such as the brush-like form but neurite branching away from the nuclei and extremely long neurites are not quantified. The ND7/23 cell line model tools can be improved for example quantifying the level of branching with the skeleton pipeline developed for the primary cortical neuron model that has as input the neurons extensions trunks, branches and endpoints.

Follow cell morphology analysis in ND7/23 cells, was performed a functional evaluation of the selected microtubule-stabilization positive candidates by assessing alterations in intracellular Ca^{2+} using Fluo-4 AM assay after drug treatment. It was expected that upon MT-stabilization calcium oscillations occur and so, higher intracellular $[\text{Ca}^{2+}]$, which would translate into higher absorbance values [213]. Indeed, the five selected positive candidates revealed functionally a preliminary indication of microtubule-stabilization effect after treatment in the ND7/23 cell line,

which is accordingly with the preliminary results obtained in ND7/23 cells cell morphology analysis.

Immortalization of cell lines such as ND7/23 was acquired at the cost of losing important features with alterations in endogenous expressions not always representing faithfully *in vivo* conditions. So, it was established a mouse primary model to better mimic neurons structures and *in vivo* environment. Besides, the choice of primary cortical neurons allowed the evaluation of MT-stability candidates in cortical neurons, cells integrated in the CNS.

The primary cortical neurons model was more sensitive to MT-stability effects showing many times transformation of growth coned into retraction bulb-like forms due to MT hyperstabilization, which translated in Aprepitant, Danazol and Disulfiram treatments causing average skeleton lengths lower than after DMSO treatment, although it was possible to visualized MT-stability morphologies in few neurons. On the other hand, Pranlukast and specially Atracurium besylate candidates displayed a MT-stability effect observed not only by cell morphology analysis but also by an average skeleton length superior to DMSO treatment.

Finally, the microfluidic device established was an easy-to-use model accomplished at a lower price and successfully enabling the culture and directional growth of primary cortical neurons generating a physical isolating of their soma and neurites through a system of interconnected channels linked by microgrooves. The PDMS polymer used for fabricating the microfluidic device is biologically inert, flexible and easily fabricated, permeable to gases, optically transparent and bonded to other materials such as coverslips easily allowing high resolution microscopy analysis.

Thus, the microfluidic model established was one step closer to a complex environment in a simple *in vitro* system appropriate for pharmacological manipulation analysing their effects in neurites separated from neuronal soma. Therefore, the evaluation of microtubule-stability candidates displayed interesting results with Atracurium besylate, Aprepitant and Disulfiram showing strong capacity for MT-stability. Still, Pranlukast treatment in de microfluidic device was inconclusive with perhaps the need for evaluation of this compound at different time points and concentrations, optimizing variables for maximum effect. As shown with the evaluation in the other established tools, Danazol shown no ability to MT modulation.

Importantly, it was possible to conclude that, in general, in any of the established tools the drugs revealed a cytotoxic effect. Positive outcomes were obtained from Pranlukast, Disulfiram, and Aprepitant drugs that demonstrate strong indications has microtubule-stability agents, although further studies on their capacity should be conducted. Since their MT modulation effects had oscillations between the different tools. Interestingly, Atracurium besylate clearly shows signs of MT-stability effect in all the *in vitro* models established, with perhaps a stability mechanism stronger than Taxol.

The top performing concentration and exposure time must be fine tuned. This is particularly relevant since it was also showed that MT hyperstabilization culminates in depolymerization compromising morphofunctional connectivity as described in the literature [213]. The MTs dynamic (stabilization, hyper stabilization, and destabilization) can be a double-edge sword [226] as demonstrated in these results. However, it should be taken into consideration that in neurodegenerative disorders the MTs are destabilized while the mammalian models have cells

with healthy and intact MTs. From a certain point it seems that the effects of these drugs in mammalian models are causing MT-destabilization, but, it is believed that in disease models these compounds will allow MTs to return to a stabilized phenotype without reaching hipper stabilization since MTs are recovering from destabilization and are not in the normal morphology evaluated in these tools.

All the conclusions regarding the MT-stability effects of the positive candidates analysed have to take into consideration that the neuronal networks from the tools established in this project have, like every *in vitro* mouse model, limited translational values and would be further improved by switching to a human system. Yet, it must be highlighted the ability of the established tools, namely microfluidic devices, in serving as valid alternatives to reduce animal experimentation.

Chapter V

BBB-on-a-chip

Introduction

The blood-brain barrier (BBB), located at the brain microvessel level, represents a dynamic and complex interface between the blood and the CNS that acts as a key player in maintaining brain homeostasis and providing protection from harmful compounds and pathogens [114, 227]. The specific properties of the BBB occur by strictly controlling the exchanges between the two compartments, blood and brain, becoming a semipermeable barrier with uniform thickness, absence of fenestrae, and negative surface charge [228].

The key characteristics of the BBB are mainly given by the microvascular endothelial cells (ECs), which are responsible for forming tight junctions (TJs) and adherent junctions (AJs), giving rise to a tight barrier. Importantly, the ECs functions in BBB formation are also supported by interactions with the basement membrane and surrounding cells, perivascular pericytes, neurons and glial cells, such as microglia and astrocytes, that all together form the neurovascular unit (NVU), Fig. 56 [227, 229].

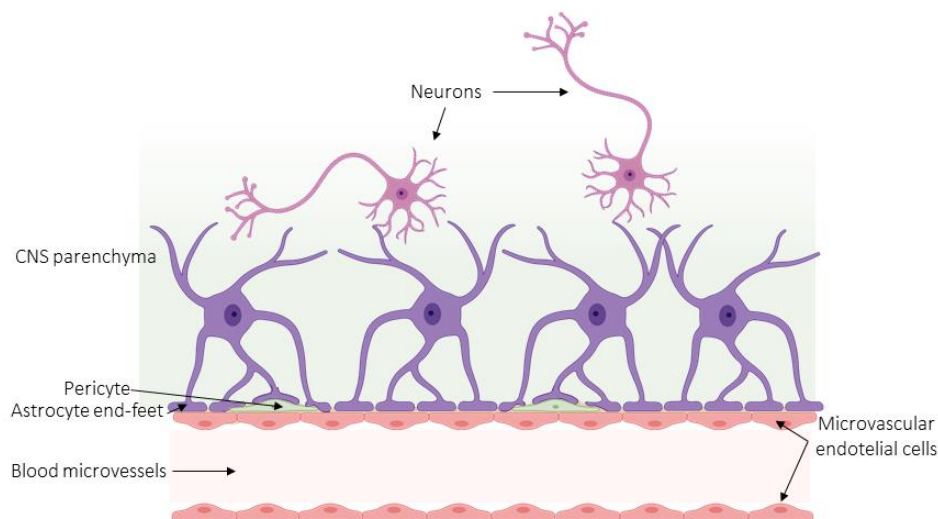


Figure 56. Blood-brain barrier (BBB) main components.

1.1. Microvascular endothelial cells

The anatomy of the BBB is mainly given by the microvascular endothelial cells [227]. Their assembly allows the selective transport mediation between blood and brain, a mechanism regulated by signalling pathways and the constituents of the TJs and AJs. Thus, molecules transport across the BBB can be by paracellular pathway (between adjacent ECs) or by transcellular pathway (through ECs). The apical region of the ECs is tight together by TJs proteins, junction adhesion molecules (JAM), claudins and occludins, being responsible for the permeability properties of the BBB since they only allow limited paracellular permeability [228]. Furthermore, below the TJs are present actin filaments, which include cadherins and catenins, that together form a line of AJs with barrier properties along with other functions: promoting ECs better adhesions, cell polarity, and regulating permeability [228].

1.2. Basal membrane

The basal membrane consists of 3 laminas, each of them composed of different extracellular matrix molecules produced by the surrounding cells: i) collagen and elastin (structural proteins), ii) fibronectin and laminin (specialized proteins), and iii) proteoglycans [227, 228]. Structurally, the basal membrane is the NVU acellular component responsible for anchoring the cells in place by surrounding ECs and engulfing pericytes, enabling their crosstalk with neighbouring cells and ensuring microvascular stability [115, 227]. Besides structural stability, the basal membrane is also fundamental for the proper placement of influx and efflux transporters in the BBB [115].

1.3. Pericytes

Pericytes have a close physical association with the microvascular endothelium providing essential support for maintenance of structural and adherent and tight junctional integrity of the BBB. The interaction of pericytes and ECs, which communicate through soluble factors, gap junctions, TJs and adhesion plaques, seem to regulate EC differentiation, proliferation and cause up-regulation of P-glycoprotein (P-gp) functional activity [227, 228].

Besides pericytes influence in BBB structure, there is evidence that these cells are also involved in transport mechanisms across the BBB and performed adjustments in response to stress conditions, as it happens in situations of prolonged O₂ deprivation [227].

1.4. Astrocytes

Astrocytes are the major glial cells of the NVU, whose extensions, known as end-feet, form the lamellae and therefore being in proximal contact with the outer surface of the capillary endothelium and with the basal membrane [227, 228].

The astrocytes have an important role in the BBB functions by promoting the synthesis of proteoglycans which increase ECs charge selectivity. These glial cells also propel neuronal function, playing a role in the link between neuronal networks and vasculatures and produce growth factors crucial for the crosstalk with ECs and other cells of NVU [115, 227]. Thereby, astrocytes can modulate the BBB characteristics without a direct physical role in it but rather providing biochemical support.

1.5. The importance of the BBB role in modelling drug development in CNS disorders

The role of the BBB as a semipermeable barrier protecting the brain is disadvantageous for therapies targeting neurological disorders. In fact, 98% of drugs are unable to pass the BBB or do so but with insufficient bioavailability [114, 227, 229].

Therefore, the ability of the pharmacological compounds to pass or not the BBB and their bioavailability should be determined during the initial stages of drug development.

Interestingly, several studies show that the BBB not only is a hindrance for drug delivery for CNS disorders but is also implicated in the pathophysiology of neurodegenerative disorders [211].

These conditions seem to present dysfunction in the BBB causing increases in permeability and suggesting a connection between BBB and neurodegenerative disorders.

Thereby, with the growing affirmation of the relation of BBB with neurodegenerative disorders, there is a significant investment in both academia and industry in the development of BBB models.

In vivo models technical difficulties, which include the high level of complexity that hamper BBB isolation, the need for a large number of animals, lengthy surgeries, laborious and costly work and are also involved in ethical concerns [230, 231]. Thus, the spotlights turn to *in vitro* models.

In vitro BBB models should be reproducible, and mimic as faithful as possible the *in vivo* BBB displaying a realistic cell architecture and possess expression of transporter mechanisms to enable the study of BBB in normal or pathological conditions, as well as evaluate BBB crossing by drugs [227]. One of the biggest advantages of the *in vitro* BBB models is their simplicity that facilitates analysis and allows larger and faster screening of drugs.

Traditionally *in vitro* BBB models rely primarily on static Transwell® inserts, a simple model in which ECs are seeded in a monolayer on top of a microporous semipermeable membrane. In these models, the insert represents the blood, the well represents the parenchymal side, and the semipermeable membrane is responsible for allowing the exchange of growth factors and small molecules but prevents the migrations of cells between compartments [211, 228, 232]. The Transwell® system can be used either with a monoculture of ECs or with co-cultures of ECs and astrocytes (and/or with pericytes).

Nevertheless, these models face some limitations, such as 2D geometry and static flow which do not properly mimic the cells nature and consequently limit BBB features [211, 232].

To overcome excess simplicity and limitations of the Transwell® systems, miniaturized microfluidics-based BBB models have been introduced as an alternative. These BBB-on-a-chip models can be developed with different designs and with mono- or co-cultures. Furthermore, these models allow the application of perfusion flow and integration of electrodes to facilitate the quantification of permeability by analysing the trans-endothelial electrical resistance (TEER) [211, 233]. Also, the double and triple co-cultures incorporate the important crosstalk between ECs and the neighbouring cells of the NVU, which allow the closest mimic of the *in vivo* conditions [227, 234].

The BBB-on-a-chip is an “*in vitro* organ chip model based on microfluidics” having the characteristics of small size, throughput and high efficiency [233]. These chips are being the beacon in brain science research. Thus, a well-validated BBB-on-a-chip would assist the development of novel drugs or methods for therapeutic delivery in the CNS and facilitate the translation to the clinics.

Therefore, in this project the first steps for the development of a BBB-on-a-chip. The aim was to establish an adequate *in vitro* model for the screening of positive candidates selected by the previously established tools, thus assessing their ability to cross the BBB.

Materials and Methods

2.1. Cell lines and culture conditions

Immortalized mouse brain endothelial cell line (bEnd.3, American Type Culture Collection, USA) were culture in a T75 flask coated overnight at 4°C with 100 µg/mL collagen type I from rate tail (C7661, Sigma-Aldrich) in 0.1M acetic acid. The medium used for cell culture was DMEM (1X) GlutaMAX™ high glucose supplemented (Gibco) with 10% (v/v) FBS (F7524, Sigma-Aldrich), and 1% (v/v) P/S. The cells were maintained in a humidified chamber in 5% CO_2 at 37°C. The medium was changed every 2 or 3 days and the cells were harvest when 80-90% confluency using 0.25% trypsin/EDTA solution with 0.05% glucose (T4799, Sigma-Aldrich).

2.2. Primary cell culture

2.2.1. Ethics Statement

All experiments involving animals were performed in agreement with institutional ethical guidelines (IBMC/INEB/i3s), the EU directive (2010/63/EU) and Portuguese law (DL113/2013). All procedures were conducted by FELASA C graded researchers.

Animals had free access to food and water and all efforts were made to minimize the suffering and the number of animals used.

2.2.2. Primary mouse astrocytes isolation and culture conditions

Primary mouse cortical cell culture was kindly provided by E. Carvalho and M. Morais, nBTT, i3s. As previously described [235], cells were isolated from Wistar Han rat pups in post-natal day 2.

First, a thorough meninges removal was performed followed by a cortex dissection. The cortex was then digested mechanically, followed by enzymatical digestion with 0.0025% (w/v) trypsin (27250018, Gibco) in HBSS without Ca^{2+} and Mg^{2+} and 0.001 mg/mL DNase I (Applichem LifeSciences) for 15 minutes at 37°C. Posteriorly, they were homogenised in DMEM (1X) GlutaMAX™ high glucose supplemented with 10% (v/v) FBS (Sigma-Aldrich), and 1% (v/v) P/S and centrifuged for 10 min at 500g. The remained pellet composed by a mixed of glial cells (MGCs) was resuspended in DMEM supplemented with 10% (v/v) FBS (Sigma-Aldrich), and 1% (v/v) P/S, followed by a filtration with a 40 µm nylon cell strainer to remove larger cell clusters. Then, the cells were plated in a T75 cell culture flask and maintained until 14 DIV. The culture medium was changed every 2-3 days.

At 14 DIV to remove the fewer adherent cells a pre-shake of the MGC culture was performed, 210 RPM at 37 °C for 2 hours. Then, detach microglia and oligodendrocyte progenitor (OPCs) the T75 flask was again shaken, 230 RPM at 37 °C overnight. The T75 cell culture flask was again incubated at 37°C, 5% CO_2 , followed by weekly shakes. After the third shake, the cells are mainly astrocytes. Nevertheless, to ensure pure astrocytes, cells were detached with trypsin and seeded again on a new T75 cell culture flask at least three times.

2.3. BBB-on-a-chip device

2.3.1. Fabrication of the model

Fabrication of the models was made together with B. Custódio, nBTT, i3s.

The 2 templates used to fabricate the BBB-on-a-chip device were designed in an open-source SCAD software (<https://openscad.org/>) or SolidWorks software and fabricated with a 3D object printer (Form 3, Formlabs, Massachusetts, USA), with a resolution of 25µm and using stereolithography technology. To fabricate the 3D models it was used Grey Resin V4 (Formlabs, Massachusetts, USA), a material with a flexural modulus of 2.2 GPa, tensile modulus of 2.8 GPa and elongation of 6.2.% [236].

The template designed for cell seeding of primary astrocytes, “BBB_astro”, was composed of a central channel with 2 cm x 1 mm x 1 mm (length x width x height), 2 feed channels with 1 cm x 1 mm x 1mm and 4 inlets with 6 mm diameter. The template for cell seeding of endothelial cells, “BBB_endo”, was composed of a central channel with 2 cm x 1 mm x 200 µm, 2 feed channels with 1 cm x 1 mm x 200µm and 2 inlets with 6 mm diameter. Both templates were based on the BBB-on-a-chip developed by Maoz, B. *et al.* [234].

After 3D printed, the templates were dipped in isopropanol solution (VWR Chemicals) for 20 minutes and were dry with argon. To finalise the curing the templates were put at 80°C overnight, and to assure the total cleaning from resin at least 3 batches of PDMS (previously described in Chapter III, section 7) were performed before the microfluidics produced were used in the experiments.

2.3.2. Fabrication of the microfluidic device

To fabricate the microfluidic device polydimethylsiloxane (PDMS) base (SYLGARD™ 184 Silicone Elastomer Base, Dow) was mixed with a curing agent (SYLGARD™ 184 Silicone Elastomer Curing Agent, Dow) were vigorously mixed at a ratio of 10:1 (w/w%), and prior to being poured into the mould the mix was degassed in a desiccator connected to a vacuum pump. The crosslinking reaction between the PDMS base and the curing agent occurred at 70°C for 2h.

When polymerized the PDMS device was then peeled off from the mould and the inlet wells (6 mm diameter) of each chamber were created using a biopsy punch (6 mm, Kai medical)

Finally, the microfluidic devices were depurated with vinyl tape (3M Vinyl Tape 471 Yellow 50mm x 33m) followed by 15 minutes of UV or 70% ethanol sterilisation.

2.3.3. Construction of the BBB-on-a-chip device

The BBB-on-a-chip consists of a microfluidic platform that supports BBB construct maintenance, with a relevant 3D model. Thus, the microfluidic device was composed of three layers (Fig. 57): i) on the bottom a microfluidic fabricated with the “BBB_endo” template; ii) in the middle a sterilised (in 70% ethanol for 15 minutes) PET membrane (ipCELLCULTURE, it4ip) with 25 mm of diameter, 0.45 µm pore size, 2.0×10^6 pore density and 12 µm thick. The porous membrane was placed in between the central channels of bottom and top microfluidics with no wrinkles;

iii) on the top a microfluidic fabricated with the “BBB_astro” template, aligned with the central channel and inlets of the bottom microfluidic.

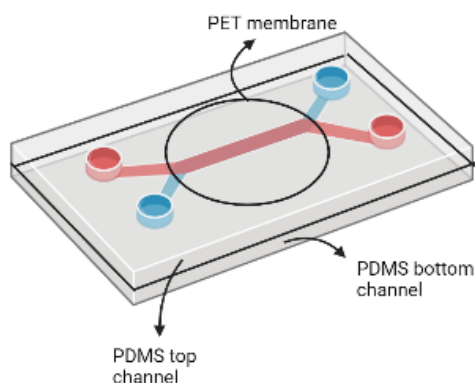


Figure 57. BBB-on-a-chip construct with three layers: i) bottom microfluidic; ii) PET porous membrane; iii) top microfluidic. Created in BioRender.com

2.3.4. Tailoring PDMS and PET membranes: increasing hydrophilicity to improve cell attachment

2.3.4.1. Plasma treatment

To increase the surface hydrophilicity the membrane was subjected to a plasma treatment prior to assembling the BBB-on-a-chip. Therefore, the PET porous membrane was placed in a petri dish into the chamber, vacuum was generated (pressure of 0.3 mbar), and air was inserted, increasing the pressure to 0.8-1.0 mbar. The air plasma was generated (at 90% intensity) and the PET membrane was treated for 3 minutes on each side. Finally, the membranes were removed from the chamber and instantly covered the petri dish to maintain sterility.

After the plasma treatment, the BBB-on-a-chip was rapidly assembled to ensure the efficacy of the treatment during coatings. All three layers were properly pressed to ensure tight contact to avoid the formation of air bubbles and to allow the proper medium flow. All the construct of the BBB-on-a-chip was made under sterile conditions.

2.3.4.2. Polydopamine coating

To increase surface hydrophilicity in both PDMS microfluidics and PET porous membrane a poly(dopamine) coating (PDA) was performed after assembling the BBB-on-a-chip platform. Dopamine hydrochloride (H8502-25G, Sigma Aldrich) was prepared at a concentration of 2 mg/mL in PBS (pH 8.5). Both top and bottom microfluidics channels, and consequently abluminal and luminal sides of PET membrane were coated with PDA solution for 24 or 48 hours at RT [237]. After PDA coating the BBB-on-a-chip device was rinse first with PBS (7.4 pH) and after with autoclaved dH₂O.

2.3.4.3. Collagen and PDL coatings for improvement of cell adhesion

The BBB-on-a-chip required specific coating for the different components: BBB-astro; BBB-endo; and PET membrane.

First, the abluminal side of the PET membrane and the top microfluidic were coated with 100 $\mu\text{g/mL}$ PDL (PDL mol wt 150,000-300,000, P1149-10MG, Sigma-Aldrich) dissolved in dH_2O ($6.7\mu\text{g/cm}^2$), overnight at 37°C .

Simultaneously, the bottom microfluidic and, consequently, also the luminal side of the membrane was coated with 100 $\mu\text{g/mL}$ collagen type I from rat tail (C7661, Sigma-Aldrich) in 0.1M acetic acid ($6.7\mu\text{g/cm}^2$), overnight at 4°C .

After each coating procedure the channels were cleansed twice with dH_2O and then filled with DMEM (1X) GlutaMAX™ high glucose supplemented with 10% (v/v) FBS and 1% (v/v) P/S to adjust the physiological pH and to equilibrate the medium flow in the channels before cell seeding.

2.3.5. Optical contact angle

In order to evaluate the effect of different methods for tailoring the BBB-on-a-chip surfaces, the contact angle in each condition was measured. After the coatings the BBB-on-a-chip platforms were rinsed, disassembled to expose the inner surface coated of the channels and dried overnight at RT. For contact angle measurements the pendant drop method was used with a 0.5 μL water droplet.

2.3.6. Co-culture of endothelial cell line and primary astrocytes

First, bEnd.3 cells (passage 3-9) were seeded on the lower channel. For that, the cells were detached from the T75 flask with 0.25% trypsin/EDTA solution with 0.05% glucose for 5-10 minutes in 5% CO_2 at 37°C . When detached the cell solution was centrifuge at 240g for 5 minutes at RT and re-suspended in DMEM (1X) GlutaMAX™ high glucose supplemented with 10% (v/v) FBS, and 1% P/S (v/v). Cell viability and density were assessed with a Neubauer chamber using trypan blue exclusion assay and cell concentration was adjusted to have 1×10^5 cells/ cm^2 . This suspension was used to filled into the lower channel. The BBB-on-a-chip device was placed in a humidified chamber at 37°C in 5% CO_2 .

After 2 hours of endothelial cell seeding, the primary rat astrocytes (passage 2-4) were added to the upper channel. Briefly, the cells were detached from the T75 flask with 0.25% trypsin/EDTA solution with 0.05% glucose for 5 minutes in 5% CO_2 at 37°C . The recovery cell solution was centrifuge at 500g for 5 minutes at RT and re-suspended in DMEM (1X) GlutaMAX™ high glucose supplemented with 10% (v/v) FBS, and 1% P/S (v/v). Cell viability and density was assessed with a Neubauer chamber using trypan blue exclusion assay and cell concentration was adjusted to have 4×10^5 cells/ cm^2 . Finally, this cell suspension was used to fill into the upper channel.

The BBB-on-a-chip device was incubated in a humidified chamber at 37°C in 5% CO_2 . Novel culture medium was added every 1-2 days.

2.4. Evaluation of cell morphology and phenotype - Immunocytochemistry

For the evaluation of morphology and phenotype of cells composing the BBB-on-a-chip, immunocytochemistry analysis was performed. For that, the medium was removed, and the microfluidic channels were fixed with 4% (wv/v) PFA for 15 min at RT. Afterwards, the cells were

permeabilized with 0.2% Triton X-100 in PBS for 10 min, at RT. The non-specific interactions were blocked with a blocking buffer (5% (w/v) FBS, 3% (w/v) BSA and 0.05% Triton X-100 in PBS) for 60 min, at RT.

The cells were then co-stained with primary antibodies in blocking buffer at 4°C overnight. Following primary antibodies, the cells were incubated with secondary antibodies diluted in blocking buffer for 60 min at RT preferably with agitation. The combination of the used primary and secondary antibodies is summarized in Table 15.

Table 15. Primary and secondary antibodies used for the BBB-on-a-chip.

Model	Primary antibody	Secondary antibody
BBB-on-a-chip	Alexa-Fluor 488® Flash Phalloidin (Invitrogen)	
	Rabbit Anti-GFAP (ab7260, Abcam)	Donkey Anti-rabbit Alexa-Fluor 647® (A31573, Invitrogen)
	Goat Anti-PECAM-1 (SC-1560, Santa Cruz Biotechnology, Inc.)	Donkey Anti-goat Alexa-Fluor 488® (A11055, Invitrogen)

Finally, the cells were incubated with Hoechst (ThermoFisher) diluted in PBS (1:10 000) for 15 min at RT. Afterwards, BBB-on-a-chips were observed under a scanning confocal light microscope (SP8, Leica, Germany).

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8, www.graphpad.com). For all datasets, tests for identification of outliers and normality were performed. To identify and remove likely outliers, the Robust Regression and Outlier Removal (ROUT) method with Q=10% was used and Gaussian distributions were performed using the Shapiro-Wilk normality test ($\alpha=0.05$). Statistical differences between groups were calculated based on two-way ANOVA (influence of two factors). A p-value below 0.05 was considered statistically significant and data are shown as mean $\pm$ standard deviation (SD).

Results and Discussion

3.1. Design and optimization of the template for BBB-on-a-chip microfluidics

The BBB-on-a-chip device developed was composed of three layers, a bottom and top microfluidic for endothelial cells and astrocytes seeding, respectively, and a PET porous membrane located in between the microfluidics mimicking the semipermeable property of *in vivo* BBB physically representing the basal membrane.

In order to produce both microfluidics used in the assembly of the BBB-on-a-chip, the first step was to design, and 3D print the templates for the microfluidics. Briefly, the object were precisely constructed with the intended channels, shapes and dimensions in a computer-aided design (CAD) software, followed by 3D printing. The initial 3D design versions were developed with code line (Appendix D) in SCAD software, a simpler open-source CAD program. However, final versions were designed in SolidWorks software (Fig. 58, (A)), a CAD software more intuitive 3D design program which is based on parametric computing creating 3D shapes from elementary geometric operations.

In Fig. 58 (B) are represented the final versions of the 3D designed templates for the production of microfluidics for the BBB-on-a-chip device. The top BBB microfluidic 3D design consists of a central channel (2cm x 1mm x 1mm) (length x width x height) linked to one feeding channel (1cm x 1mm x 1mm) on each side that in turn are connected to an inlet (6 mm diameter), on the opposite corners of these inlets are other two inlets (6 mm diameter), and this structure is surrounded by a frame. The bottom microfluidic 3D design has a similar pattern with a central channel (2cm x 1mm x 200µm) linked to one feeding channel (1cm x 1mm x 200µm) on each side that is also connected to an inlet (6mm diameter), this structure is also surrounded by a frame. The central and feeding channel dimensions were adopted from the BBB-on-a-chip developed by Maoz, B. *et al.*[234] and the dimensions of the inlets were adapted to the biopsy punches available in the lab. The differences in height of the channels, comparing both designs, were made wittingly so that when mounting the BBB-on-a-chip the channels from the top microfluidic would be higher, allowing the seeding of the endothelial cells around the channels to form a vessel-type structure. Finally, two inlets were added to the design of the top BBB microfluidic to allow direct access to the lower channels from above when the device was assembled.

After the 3D design, the file was transposed to an SLD file domain to be transferred to the 3D printer (Fig. 58, (C)) program PreForm by FormLabs®. The templates were printed with a grey resin and the time of printing was 6-8 hours for 2 replicates of each template. Cleaning of the mould was achieved by submerging the mould in isopropanol (10-15 min), let it dry, and post-printing process at 80°C, overnight for a better solidification. In Fig. 58 (D) it is possible to detect the differences of height in the channels of both moulds', indication of a correct 3D printing. Nevertheless, after producing the first PDMS microfluidics they were sticky indicating an insufficient time of curing. The differences between the devices used in Chapter IV and the 3D printed moulds might arise from the resin used, as its composition is undisclosed. The challenge was overcome after 3 rounds of microfluidics batches which might indicate the presence of

some resin debris in the mould. So, in every new template version only after the third batch, the microfluidic was used for BBB-on-a-chip construction.

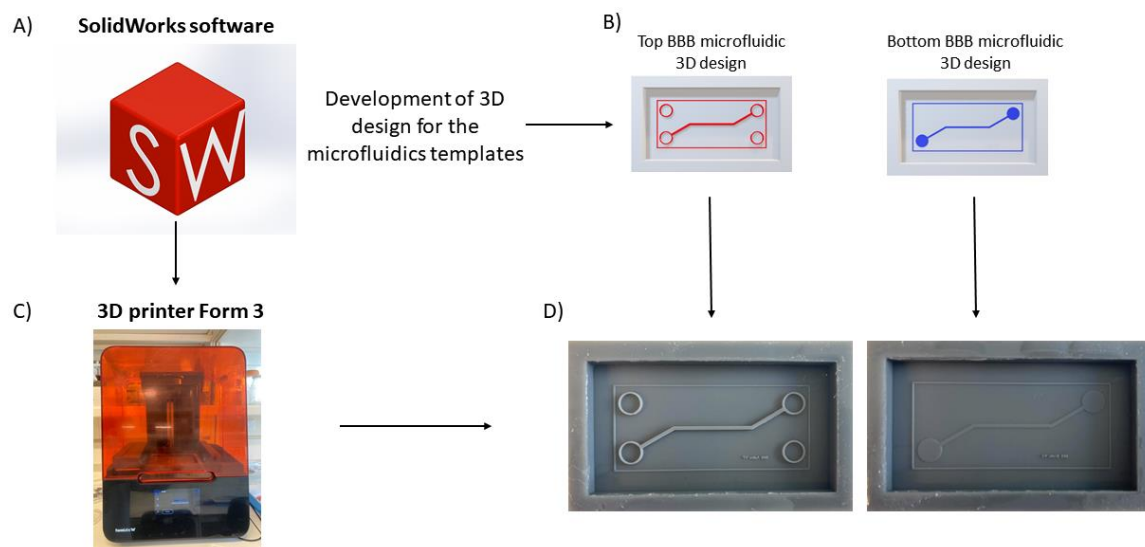


Figure 58. Final process of design the template for BBB-on-a-chip microfluidics (bottom and top). A) SolidWorks software used in the development of computer 3D design. B) 3D design of the final versions for each microfluidic: top BBB microfluidic 3D design in red and bottom BBB microfluidic 3D design in blue. C) 3D printer used in the process, Form 3, Formlabs®. D) Final 3D printed moulds to serve as templates for microfluidics.

The 3D printing technology allowed the inclusion of a reproducible “custom-made” design with various optimizations (see in detail in Appendix D, Table D1) made in the lab as the project evolved, an advantage over commercial fabrication, with the addition of lower time and cost.

3.2. Tailoring PDMS and PET membranes: increasing hydrophilicity to improve cell attachment

The choice of PDMS for the polymer used in the production of microfluidics has several advantages, including optical transparency facilitating imaging analysis, biocompatibility, ease to use and low price [216]. However, PDMS surfaces are inert and very hydrophobic since after the polymerization of PDMS by crosslinking with curing agent, the surface of the microfluidics has hydrocarbon groups ($-C_xH_y$) exposed, mainly ($-CH_3$) [238]. Consequently, the use of PDMS microfluidics within the BBB-on-a-chip required tailoring the PDMS surface for the cell adhesion to occur. Furthermore, PET porous membrane located in between microfluidics in the BBB-on-a-chip platform was also expected to be highly hydrophobic, requiring surface tailoring prior to cell seeding for uniform adherence and proliferation.

The analysis of a surface energy and affinity of a liquid (e.g. pure H_2O) toward a substrate can give a simple and effective method to study the surface properties, such as wettability/hydrophilicity [239]. The contact angle technique measures the “ability of a liquid to wet the surface of a solid” [240]. The shape that a drop acquires on a surface is related with surface tension and nature, the more the droplet is repelled by the surface, higher is the contact angle.

Therefore, different surface modifications were tested in the BBB-on-a-chip device, and the contact angles were measured on PET membrane and on both microfluidic components to

evaluate the surface wettability after each treatment (Appendix E, Table E1). Although the same material was used, the channels have different heights which can interfere with surface treatments and the measurements.

Fig. 59 shows the mean contact angle measurements results. The PDMS channels from the bottom and top microfluidic had a mean contact angle of 93.6° and 99.7°, respectively, confirming the polymer high hydrophobicity (contact angle > 90°). Surprisingly, the PET membrane had a contact angle around 69.6° which, contrary to theoretical expectations points to a more hydrophilic surface (Fig. 59 (B)).

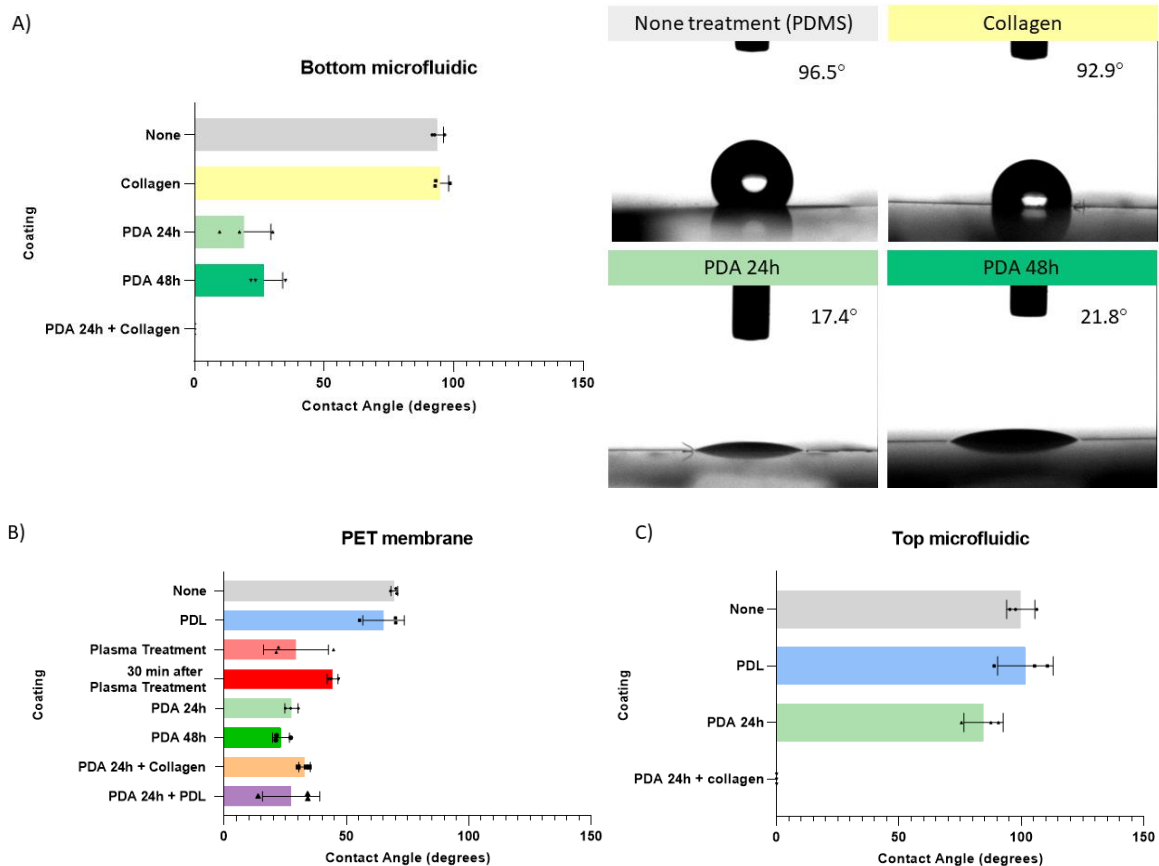


Figure 59. Contact angle measured using the pendant drop method. A) Analysis of coating methods evaluated for endothelial seeding in PDMS bottom microfluidic channel. B) Analysis of coating methods evaluated for astrocytes seeding in PET membrane. C) Analysis of coating methods evaluated for astrocytes seeding in PDMS top microfluidic channel. n=1 independent experiment with three contact angle measurements *per* condition, Mean±SD.

One tailoring approach tested was to coat the PDMS surface and the PET membrane with an ECM-based component by physical adsorption (Fig. 60). It is commonly described in the literature and used in a Transwell® model established in the lab [231, 241]. For the culture of bEnd.3 endothelial cells and primary rat astrocytes it is required a pre-coating of 100 µg/mL type I collagen and 100 µg/mL PDL respectively, for improvement of cell adhesion. However, coating with collagen on the bottom microfluidic revealed a contact angle of approximately 94.9° (Fig. 59 (A)). Thus, after collagen coating the hydrophobicity of PDMS surface was similar to the hydrophobicity pre-collagen coating, which may indicate that collagen is not being able to be adsorbed at the channel surface. In its turn, the same effect is observed with PDL coating on the PET membrane and the top microfluidic, with a mean contact angle of 69.4° and 101.6° (Fig. 59

(B, C)). Indeed, as shown in Fig. 63 (A), with only PDL and collagen coating, although there was cell adhesion of both endothelial cells and astrocytes the cell distribution was lower than desired and both cell types demonstrated signs of stress. Primary astrocytes showed an activated morphology although with some level of stress, however this morphology needs to be confirmed by further analysis of different astrocytes cytoskeleton structures. The endothelial cells were more rounded and not elongated, demonstrating an altered morphology when compared to the endothelial cells' phenotype in the BBB. Importantly, bEnd.3 cells, although, with signs of stress, managed to form a cell layer to grow around the channel. The stress and lack of cell adhesion demonstrated by the co-culture was perhaps an indicator of inadequate surface coating. Given the high hydrophobicity demonstrated by the PDMS microfluidics, the collagen and PDL adsorption effect was not being achieved.

Furthermore, to improve the wettability of PDMS by collagen and PDL, it was evaluated the traditional technique of plasma treatment.

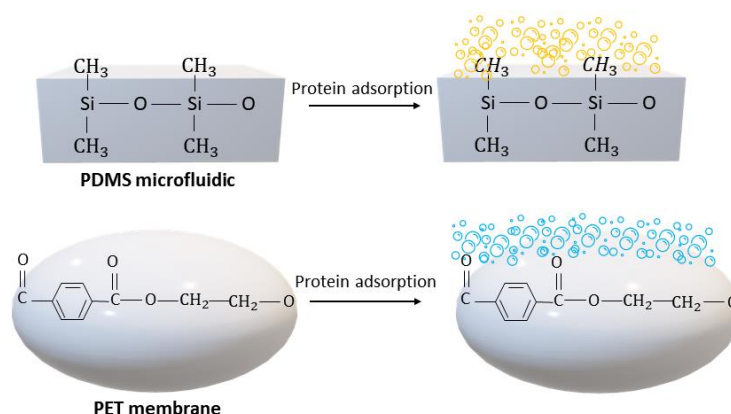


Figure 60. Schematic example of protein adsorption effect on PDMS and PET surfaces. In yellow and blue are represented the proteins adsorbed, collagen and PDL, respectively.

The plasma treatment technique promotes exposure of reactive groups due to electrical charges that collide with the surfaces. It causes oxidation and formation of silanol groups ($-\text{SiO}_x$) in PDMS (Fig. 61) and is also described in the literature as capable of increase the PET surface hydrophilicity [238, 242]. Due to the assembly of the BBB-on-a-chip device, it was not possible to performed plasma treatment on both microfluidics and PET membrane because they would repel each other disabling the attachment of the components. Since the PET membrane does not cover entirely the microfluidics area it was not possible to perform plasma treatment on both microfluidics since they would still repel each other on the edges. So, plasma treatment was only performed in the PET membrane to improve its wettability. The mean contact angle measured of 29.4° proves the higher hydrophilicity of the membranes after plasma treatment (Fig. 59 (B)). However, the large standard deviation associated indicates that the treatment was not achieved uniformly. Furthermore, surface modifications associated with plasma treatment are highly reversible with the PDL and collagen coatings having to be performed immediately to maximize the adsorption effect. In fact, the contact angle 30 minutes after plasma treatment increased to 44.3° (Fig. 59 (B)). As demonstrated in Fig. 63 (B) collagen and PDL coating after plasma treatment improved cell adhesion of both endothelial cells and astrocytes in the membrane area when comparing with direct adsorption of PDL and collagen on PET, condition previously tested (Fig. 59 (A)). Regardless, adherent endothelial cells were still in low number

and were not yet completely covering the channel surface, visible in the 3D construct of Fig. 63 (B). Not surprisingly since wettability was only improved in the membrane area and not in the PDMS, limiting the collagen adsorption in the microfluidic channels, the endothelial cell adhesion was compromised. Still, the plasma treatment in the PET membrane allowed a better adhesion of primary astrocytes (Fig. 63 (B)) displaying a star-like shape as expected from healthy astrocytes. Nevertheless, it was possible to see by the 3D construct that astrocytes adhesion did not occur uniformly in the membrane area, perhaps a consequence of uneven plasma treatment, as mentioned before.

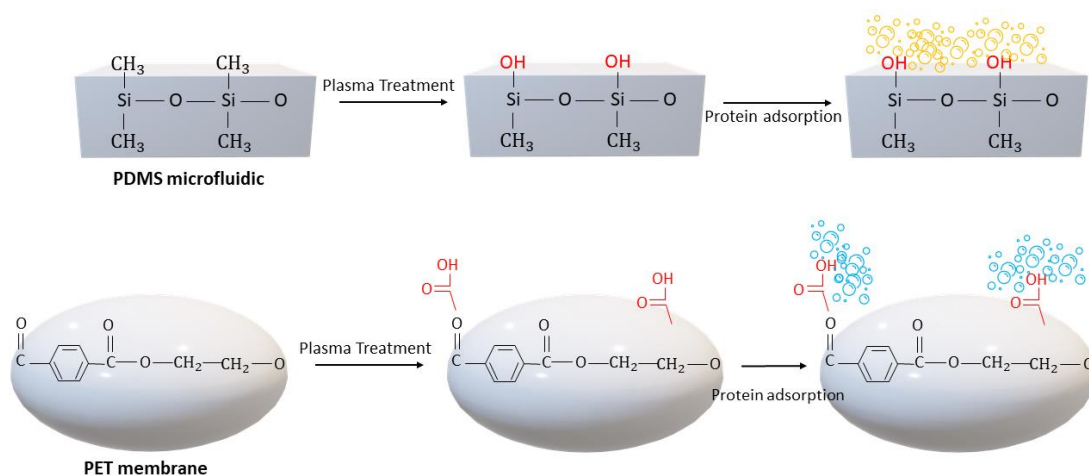


Figure 61. Schematic example of plasma treatment effect on PDMS and PET surfaces, followed by a step of protein adsorption. In red are represented surface modifications induced by plasma treatment and in yellow and blue are represented the proteins adsorbed, collagen and PDL, respectively. [238]

Finally, it was evaluated a theoretically favourable surface modification that would allow the covalently bond of ECM proteins resorting to a linker. The aim was to overcome the shortcomings of other surface modification techniques while maintaining the benefit of using coatings of ECM proteins and, for that, the method used was to coat both PDMS microfluidics and PET membrane with PDA.

Dopamine diluted in an alkaline solution (in PBS pH 8.5) undergoes an oxidative polymerization forming PDA layer (Fig. 62) and it is described in the literature as capable of promoting cell adhesion by itself or by working as a linker between ECM proteins and PDMS and PET surfaces [237, 243]. Using the contact angle technique, the incubation time of PDA was optimized in the BBB-on-a-chip construct. Furthermore, it was evaluated the capacity to increase the wettability of PDMS and PET surfaces of PDA by itself and PDA followed by collagen or PDL coating.

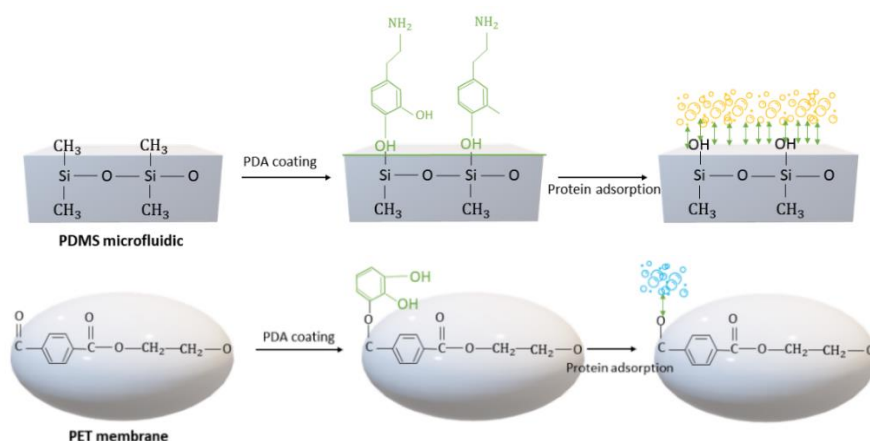


Figure 62. Schematic example of polydopamine (PDA) coating effect on PDMS and PET surfaces, followed by a step of protein adsorption. In green are represented surface modifications induced by PDA coating and in yellow and blue are represented the protein adsorbed, collagen and PDL, respectively. [244]

First, for the evaluation of PDA coating alone in the PDMS bottom microfluidic and PET membrane it was assessed two incubation times, 24h and 48h [237]. As demonstrated in Fig. 59 (A, B), after 24h and 48h incubation the mean contact angle was approximately 19.1° and 26.8°, respectively, in PDMS lower microfluidic and 27.5° and 23.2°, respectively, in PET membrane. Proving a shift of PDMS and PET to a hydrophilic feature as intended. Since 24h incubation of PDA was enough for surfaces modifications this time point was maintained for the other conditions.

Afterwards, it was assessed the effect on the surface wettability of PDA linked to collagen and PDA linked to PDL on both PDMS microfluidics and PET. In the case of the bottom microfluidic, the contact angle was measured for “PDA 24h + collagen” since the aim was to have endothelial cell adhesion, and the result was $\cong 0^\circ$ (Fig. 59 (A)). Hence, revealing extreme hydrophilicity that unable the measurement on any contact angle replicates due to water drop spread. In the PET membrane, two conditions were tested “PDA 24h + collagen” and “PDA 24h + PDL” since both cell types would be in contact with the membrane. A contact angle of 32.9° and 27.4° were obtained, respectively, maintaining the hydrophilicity surface detected after PDA treatment in both conditions. However, PDL coating after PDA treatment demonstrated a high standard deviation which can indicate an improper coating. Finally, due to the different heights in the channels of both microfluidics it was also analysed the condition “PDA 24h + PDL” on the top microfluidic (Fig. 59 (C)) revealing a mean contact angle of $\cong 0^\circ$, hydrophilicity surface.

Although it was demonstrated through the contact angle technique that formation of PDA layer in both PDMS and PET membrane improved the wettability of the BBB construct, these results do not translate in successful cell culture in the BBB-on-a-chip, as shown in Fig. 63 (C, D). Indeed, the adherence of bEnd.3 endothelial cells was verified with higher number of cells in the PDMS channel (Fig. 63 (C, D)), but they still had signs of stress not acquiring the elongated morphology. Since cell adhesion was clearly improved with the PDA treatment. According to the literature is not expected toxicity effects in endothelial cells, so the altered morphology observed can perhaps be due to different variables other than coating, such as stress caused by highly confluent cells. However, these are preliminary results and more studies with different basal membrane proteins (e.g. fibronectin) need to be performed to corroborate these results and

withdraw conclusions. Nevertheless, it will be important to optimize bEnd.3 cells density by evaluating lower seeding densities.

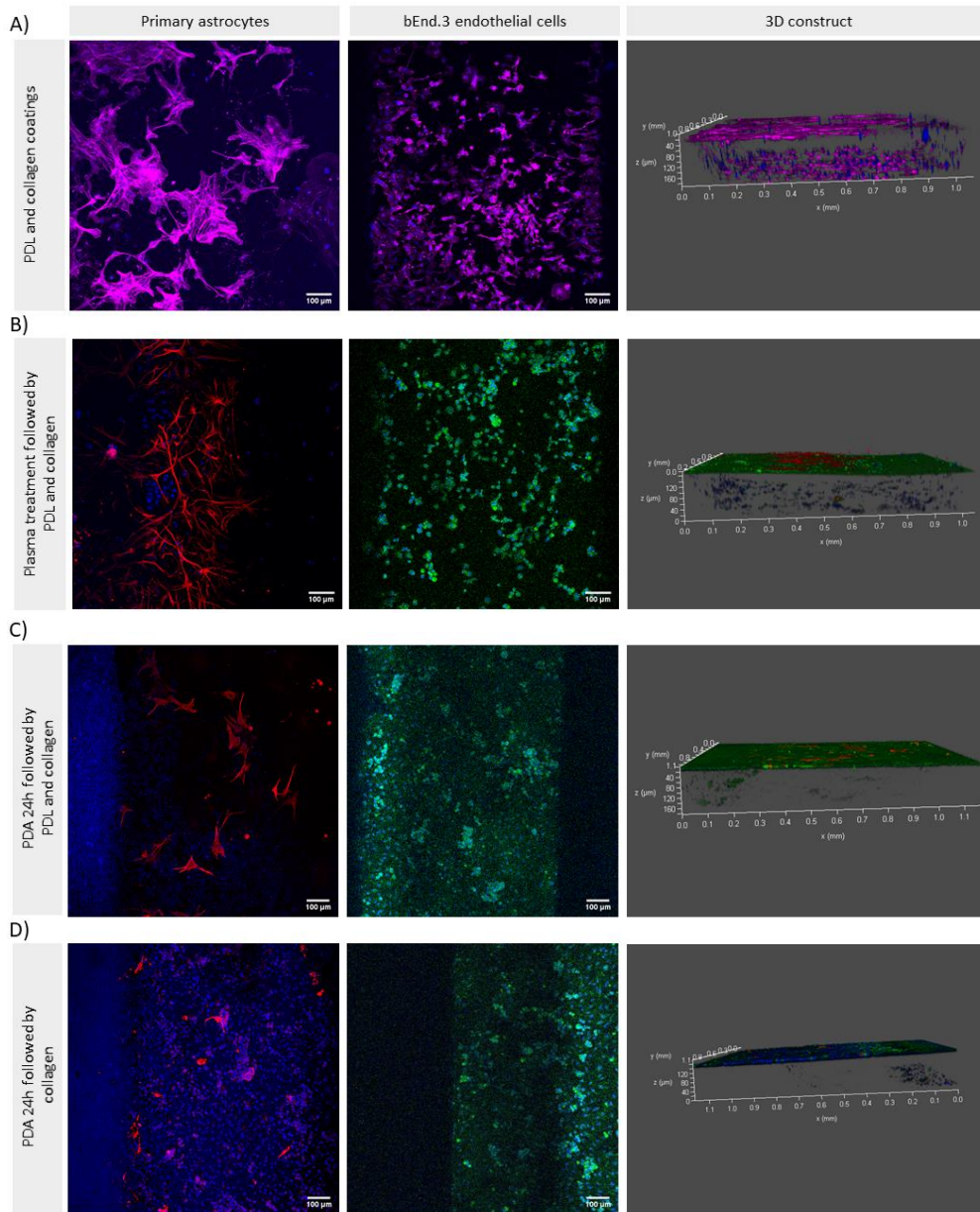


Figure 63. Representative images of acquired channels in confocal microscope of BBB-on-a-chip construct. Images on the left show the astrocytes in the PET membrane, images in the middle show the endothelial cells at the bottom of the channel and images on the right are a 3D reconstruction. (A) 100μg/mL PDL and 100μg/mL collagen coatings without pre-treatment; (B) Plasma treatment followed by 100μg/mL PDL and 100μg/mL collagen coatings; (C) 2mg/mL PDA treatment followed by 100μg/mL PDL and 100μg/mL collagen coatings; (D) 2mg/mL PDA treatment followed by 100μg/mL collagen coating. Phalloidin (pink), GFAP (red), PECAM-1 (green) counterstaining with Hoechst (blue). Scale bars: 100μm.

On the other hand, the astrocytes react differently to the PDA treatment. First, to the best of our knowledge, no literature reports the combination of PDA coating linked to PDL. However, through the analysis of the molecule structures it was not clear if they would chemically bond. At a basic environment provided by the cell medium (pH 7.4) PDA exposes chemical groups that, although can link to collagen, would not link to PDL molecule. However, the contact angle

measurements (Fig. 59 (B)) revealed a hydrophilicity feature in the “PDA + PDL” coating condition raising the doubt about the link between PDA and PDL, with the chance of the rinse performed after PDA treatment with PBS and dH₂O lowered the pH enough to allow PDL binding. Therefore, it was tested in the BBB-on-a-chip device the astrocytes culture with PDA followed by PDL (Fig. 63 (C)) and solely PDA coating (Fig. 63 (D)). In both conditions the adhesion of astrocytes was much lower than the observed after plasma treatment. But the condition with the combination of PDL with PDA showed an increased adhesion of astrocytes when compared with PDA alone. Nevertheless, the few astrocytes present were stressed, and the cells that adhere only to the PDA did not exhibit the characteristic cell extensions (Fig. 63 (D)). Considering the results in the contact angle technique it should be pointed out that the lack of astrocytes adhesion probably was not by failure of PDA in improving PET membrane wettability but perhaps because of some chemical effect that PDA layer is causing on the primary astrocytes. Dopamine (DA) is a naturally occurring neurotransmitter and would be expected that it can interfere with the astrocytes, by altering artificially their behaviour (positive or negative outcomes) [245]. Nevertheless, the layer formed for tailoring the surfaces was PDA and the polymerization of DA to PDA is a fast irreversible reaction at a basic pH 8.5. In the unlikely scenario of DA remained in solution, it would be polymerized due to the medium culture pH and the presence of oxygen [245]. Hence, no DA would be present in solution to interfere with astrocyte physiology. Additionally, PDA layer is not described as toxic to astrocytes [245]. Another hypothesis for the astrocytes stress-like morphology can be the formation of aggregates during self-oxidation of free DA to PDA. This reaction caused the formation of precipitates which conferred a black tone to the membrane and PDMS. Considering all, we plan to decrease the time of exposure to the PDA or even its concentration to avoid the accumulation of precipitates. Additionally, considerations on the cell density and time of seeding should be studied.

Conclusion

Therapies targeting neurological disorders are hampered by the selective nature of the BBB and there is a continuous effort to improve BBB models. This project shows the initial steps for the set-up of a BBB-on-a-chip device towards the implementation of a relevant model for evaluation of drugs permeability into the CNS.

The BBB-on-a-chip, composed by 2 microfluidics and 1 porous PET membrane, was produced by a “custom-made” approach. The use of 3D printing technology provided several advantages over commercial options by successfully allowing the creation of microfluidics in a uniform, low cost and reproducible manner. The channels and inlets were optimized by tuning the dimensions and complexity. The use of PDMS was based on its widely use in biomedical applications due to polymer their biocompatibility, optical transparency, low price and ease of use, allowing the development of a cost-effective BBB-on-a-chip model.

The biggest challenge on the development of the BBB-on-a-chip device was tailoring PDMS and PET surfaces to improve cell adhesion. None of the 3 approaches tested showed the expected efficacy in cell seeding. ECM-proteins adsorption might have been insufficient since PDMS hydrophobicity impairs their adsorption. Despite having managed to form a cell layer throughout the channel, the cells exhibit a stress-like morphology. ECM proteins coatings better mimic *in vivo* scenario. Therefore, the addition of pre-coating treatments to improve surfaces hydrophilicity, proteins adsorption, and consequently cell adhesion should be maintained in future experiments.

Regarding the pre-coated treatments performed during this project, the traditional plasma treatment presented several limitations due to the shape of the commercial PET membrane that did not cover the microfluidic devices. The, plasma treatment seems to reduce the stress-like astrocyte morphology. The formation of a PDA layer was tested as a promising approach to increase wettability by establishing a physical link with PDMS or PET. Astrocyte's phenotype to PDA surfaces was discouraging. Although, to the best of our knowledge, there are no reports of toxicity caused by PDA in astrocytes and endothelial cells, only a few adhere to the membrane and show stress-like morphology. Nevertheless, these pilot experiments open new avenues to the establishment of coating pre-treatments.

Several *in vitro* models with flow, reported the loss of ECM protein coating, even after plasma treatment. This is a reversible coating hence the ECM proteins may be washed out by the medium flow, resulting in cell detachment.

In the future it would be important to confirm that the link between PDA and collagen protein is effectively occurring and to optimize the selected tailoring approaches. Namely, PDA concentration and incubation times to prevent precipitates formation detected after PDA treatment, which can be harmful for the cells, and homogeneous plasma treatment through all the membrane surface.

The BBB-on-a-chip developed in this project it is still in its infancy and only preliminary steps were performed. However, when completely established this *in vitro* model will be one more

tool to proceed with the evaluation of the positive candidates to microtubule-stability agents, allowing to study their ability to cross the BBB.

Chapter VI

General conclusions and future considerations

Neurodegenerative disorders encompass a wide range of debilitating conditions without therapeutic options to tackle progressive degeneration and/or death of neuronal cells. Every year millions are diagnosed with these disorders worldwide and the development of alternative drugs to reduce such burden is an urgent need.

Neurons are particularly regulated by MTs dynamics, so, unsurprisingly dysregulation of this cytoskeleton component have been associated with the onset of different neurodegenerative disorders. Indeed, *in vivo*, and *in vitro* studies have identified the loss of MTs mass and MT dysregulation, namely altered MT-stability, in various unrelated neurodegenerative diseases [3-5]. The disclosure of MTs-destabilization role in neurodegeneration propels the development of MT-stabilizing to restore homeostasis.

Recently, drug repurposing [246] has emerged as a strategy to identify new targets for approved drugs outside of their original applications to accelerate new treatments to the bedside. One example is the drug Fingolimod, initially developed for transplant rejection, which has now been repurposed for the treatment of multiple sclerosis [247]. One of the main advantages is the existence of proven bioactivity and safety in humans.

This project proposes an elegant but simple model to unravel the complexity of the nervous system through the establishment of fungus *U. maydis* has a whole-organism high-throughput platform suitable for drug screening.

We took advantages of the phenotypically similarities between *U. maydis* and neurons to screen a Prestwick's chemical library for MT modulation compounds. First, an *in silico* analysis was performed on the top performed 22 hits. The psychochemical properties, therapeutic classes and effect, brain disorders related studies and clinical trials concluded or on going, were assessed. Five drugs were selected to pursue with further validation in mammalian systems. The drugs Aprepitant, Atracurium besylate, Danazol, Disulfiram and Pranlukast were selected because they represent an overview of the positive hits therapeutic classes and effects.

After, the aim was to validate the potential hits in mammalian models with different degrees of complexity. Considering the wide range of neurodegenerative disorders, the neuronal models established in this project were ND7/23 cell line, primary cortical neurons and microfluidic device. The models cover both CNS and PNS and make the most of the best characteristics of immortalized cell models and primary cell models.

ND7/23 cell line model was established has a tool for evaluation of MTs-stability agents resorting to differentiated ND7/23 cells and analysing the compound effect in their unbranched outgrowth neurites by using cell morphology analysis and "collar occupancy" method after drug treatment. For one lever closer of *in vivo* conditions a primary cortical neurons model was also established for evaluation of promising microtubule-stability drugs, where cell morphology and average skeleton length readouts for MT modulation. Lastly, relevant *in vitro* model the mouse primary cortical neurons were cultured into a 2.5D environment. The microfluidic device allowed for physical separation of neurons soma and axons extensions for further pharmacological treatment assess the MTs stability effects. All the mammalian models developed in this project were accomplished at the expense of drug controls, Nocodazole (a MT-

destabilizer), Taxol (a MT-stabilizer) and DMSO (drug solvent) that were further used as a comparison in the evaluation of positive candidates.

Evaluation of Danazol in ND7/23 cells model did not allow withdraw clear conclusions. Moreover, neither in 2D primary cortical neurons model or microfluidic device Danazol revealed the capacity to induce MT-stability. However, the other hits revealed some promising and exciting results. Aprepitant, Danazol, Disulfiram and Pranlukast exhibit preliminary indicators of MT-stability effects. To be noted, that some variations in the cellular response was observed in the different models. So, it will be important to fine-tune some conditions such as concentration and time of treatment. Interestingly, Atracurium besylate unveils an excellent microtubule-stability effect in all the *in vitro* models established. Overall, 4/5 positive hits extrapolated from *U. maydis* HTS revealed as possible microtubule-stability agents.

Thus, *U. maydis* filamentous form model together with drug repurposing proposes a faster and viable solution for microtubule-stability drug development.

Nevertheless, it would be important to quantify the number of apoptotic cells after drug treatment to assess the effect of these MT modulations in cell viability. As an example, flow cytometry analysis using Cy5 Annexin V, a probe sensitive for apoptotic cells since it binds to negative charged phospholipid surfaces with a higher affinity for phosphatidyl serine than other phospholipids would be a proper method for quantification of % apoptotic cells. Furthermore, it would be equally important to analyse the neuronal cells viability. Presence of actin in the culture is a sign of viable and healthy cells, therefore tracking actin behaviour would be an option to study neuronal viability.

One important step in drug development for neurodegenerative diseases is BBB permeability and brain retention capacity to the compounds. Therefore, would be important to determine the ability of drugs to pass the BBB during initial stages of drug development and so, a BBB-on-a-chip device was preliminarily developed in this project.

The BBB-on-a-chip developed was construct resorting to 2 PDMS microfluidics on top of each other with a PET porous membrane. The use of a 3D printing technology to design and build the microfluidic templates allowed the development of a “custom-made” methodology at lower cost and easy modified for design optimizations.

Tailoring the PDMS and PET surfaces for improvement of cells adhesion was the biggest challenge and 3 approaches were studied: ECM-protein adsorption, plasma treatment, and PDL coating. Preliminary results obtained from each of the tailoring approaches were conditioned by physical limitations presented by the form and size of a porous membrane (the only commercialized item of BBB-on-a-chip construct). Nevertheless, the best approach for modifying the surfaces of this BBB-on-a-chip device would be joining tailoring with plasma treatment followed by PDL coating in the PET membrane and PDA treatment followed by the collagen coating in the lower microfluidic.

A well-established BBB-on-a-chip would assist the development of microtubule-stability agents and other pharmacological drugs or methods for therapeutic delivery in the CNS.

Besides the contribution of *U. maydis* HTS model as a faster bench to bedside transition, the filamentous fungus form can be used in mechanistic studies untangle the complexity network behind axonal pathologies. In order to ground even more the *U. maydis* similarities with human neurons it will be interestingly, in the future, performed comparative studies of transcriptomics of *U. maydis* and axons and also use it to be able to unravel key pathways of other cytoskeleton components (e.g. actin) on modulation and for high-throughput screening for drugs.

This project validates *U. maydis* has a “SIMPLER: Fungi-to human” approach and points out the promising future applications of this filamentous fungi.

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Appendix A

Evaluation of *Ustilago maydis* high-throughput screening results

In the following section is present an explanation of *U. maydis* hits, an extended analysis of HTS results, namely the distribution of Prestwick chemical library compounds per therapeutic effect, the evaluation of the negative hits, i.e. bioactive compounds that showed microtubule destabilization properties in *U. maydis* model, and the interconnection between therapeutic effects of positive hits. In table J is presented a more extended analysis of the physicochemical properties of positive candidates together with other therapeutic effects reported in the literature relevant for the neurodegenerative area.

Drug screening in *U. maydis* using Prestwick compound library was conducted through 4 separate screenings of 4 plates (96 wells) each. The fungal cells were exposed for 4 hours to the different compounds, and after the OD_{600nm} and the fluorescence (488nm – 590nm) were measured in a multi-mode plate reader (BioTek SynergyMx™). During the screening it was observed an edge effect, this can be due to the: plates; dispensing cassette; or pipetting errors. After trying different approaches, we concluded that this effect was observed in the different set-ups. So, we used a sophisticated and widely used normalization method, B-score [248]. The B-score corrects biases within plates, rows and columns. It involves a two-way median polish and a smoothing function. In this manner, the normalization starts with units of fluorescence *absorbance. From here a average of the surrounding edges is calculated to overcome the edge effect. And lastly, the B-score normalization corresponds to the B-score * (RFU*Absorbance) for each well. The positive hits are retrieved if the value is higher than Mean + 3*SD, and a negative hit is below Mean - 3*SD. This normalization allowed cherry picking the top-performer candidates. In Fig. A1 an example of one of the screens performed.

	1	2	3	4	5	6	7	8	9	10	11	12	RFU*OD
A	479,925	618,844	588,546	618,45	578,151	615,515	582,622	538,067	528,374	517,643	539,76	550,592	
B	498,52	587,51	659,581	572,378	560,67	533,983	526,05	525,72	489,419	497,354	512,925	517,293	
C	530,53	594,018	574,035	560,091	556,5	516,275	516,664	527,334	486,095	517,979	506,66	467,019	
D	527,73	562,548	601,524	546,93	530,162	535,389	650,752	508,464	518,742	528,63	317,772	480,891	
E	519,414	553,175	545,359	537,664	491,028	510,435	507,744	498,848	502,64	482,832	496,228	459,648	
F	552,26	594,405	536,718	528,496	481,635	467,983	503,43	500,58	546,592	486,563	499,269	475,308	
G	557,061	514,792	540,918	509,124	484,803	516,304	505,98	473,76	490,78	501,375	469,165	493,028	
H	506,328	500,256	502,86	533,025	492,285	500,408	485,05	476,157	485,46	502,911	481,096	513,086	

	1	2	3	4	5	6	7	8	9	10	11	12	B-score
A	558,682	588,028	639,0155	575,2645	583,0925	558,3025	532,0585	527,047	503,531	518,557	531,7585	517,293	
B	532,655	592,7408	580,6173	597,198	560,253	552,1275	539,7473	520,2175	509,3858	509,4915	515,2668	510,1787	
C	540,089333	563,6558	603,8035	562,4608	539,2995	535,634	555,1028	509,2358	513,3685	504,6848	453,9238	501,6147	
D	537,497333	569,1118	557,218	557,3603	532,4618	551,906	517,0653	548,919	506,4573	459,3313	503,1023	414,813	
E	544,388333	555,4315	557,2703	527,9533	514,974	500,536	540,8663	504,857	511,7535	503,5153	439,8803	484,1423	
F	556,96	539,2363	552,2945	516,2853	493,0775	502,951	495,5718	505,6575	495,1408	507,517	481,816	483,9817	
G	524,46	548,16	515,8735	521,8105	499,837	489,7935	494,636	493,3743	501,7968	487,3548	493,692	485,853	
H	528,6585	507,9933	524,733	501,423	506,0787	497,8797	494,1817	481,4233	489,9493	489,3103	495,054	487,062	

	1	2	3	4	5	6	7	8	9	10	11	12	B-score normalization
A	0,85903072	1,052406	0,92102	1,075071	0,991525	1,102476	1,095034	1,020909	1,049338	0,998237	1,015047	1,064372	
B	0,93591537	0,991175	1,136	0,958439	0,982895	0,967137	0,974623	1,010577	0,960802	0,976177	0,995455	1,013945	
C	0,98230046	1,053867	0,950698	0,995787	1,031894	0,963858	0,930754	1,03554	0,946873	1,026342	1,116179	0,931031	
D	0,98182813	0,988467	1,079513	0,981286	0,995681	0,970073	1,258549	0,926301	1,024256	1,150869	0,631625	1,159296	
E	0,95412405	0,995937	0,978626	1,018393	0,953501	1,019777	0,938761	0,988098	0,982192	0,958922	1,128098	0,949407	
F	0,99156133	1,102309	0,971797	1,023651	0,976794	0,930474	1,015857	0,989959	1,103912	0,958713	1,036223	0,982079	
G	1,06216108	0,939127	1,048548	0,975688	0,969922	1,054126	1,022934	0,960245	0,978045	1,028768	0,950319	1,014768	
H	0,95776007	0,984769	0,958316	1,063025	0,972744	1,005078	0,981522	0,989061	0,990837	1,027796	0,971805	1,053431	

SD	0,07118	1,215332	Hit>AVG+3*SD
Mean	1,0017	0,788219	Hit<AVG-3*SD

Figure A1. Screen performed in *U. maydis* using Prestwick library. An example of B-score normalization is shown. This statistical method was performed to overcome the edge effect and to retrieve the stronger candidates. Positive hits > Average + 3*SD; Negative hits < Average – 3*SD. The compound on D7 is a positive hit, and D11 is a negative hit.

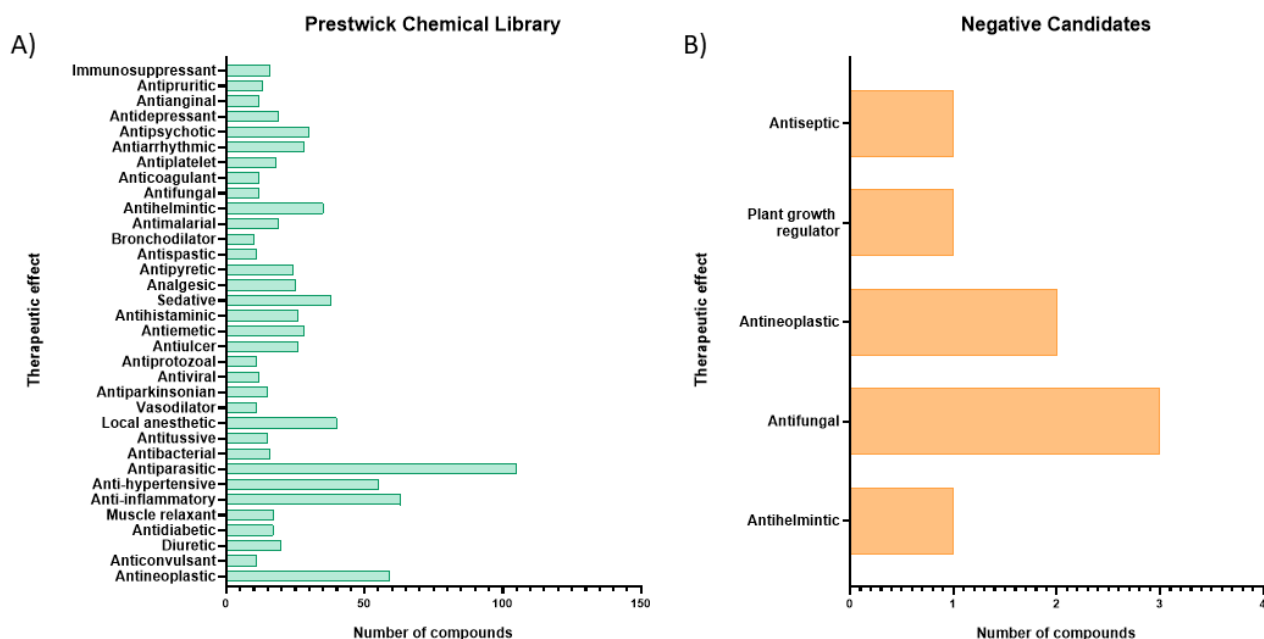


Figure A2. Distribution of compounds per therapeutic effect. It must be taken into consideration that each drug can be included in more than one therapeutic effect group. A) Prestwick Chemical Library; B) Negative Candidates.

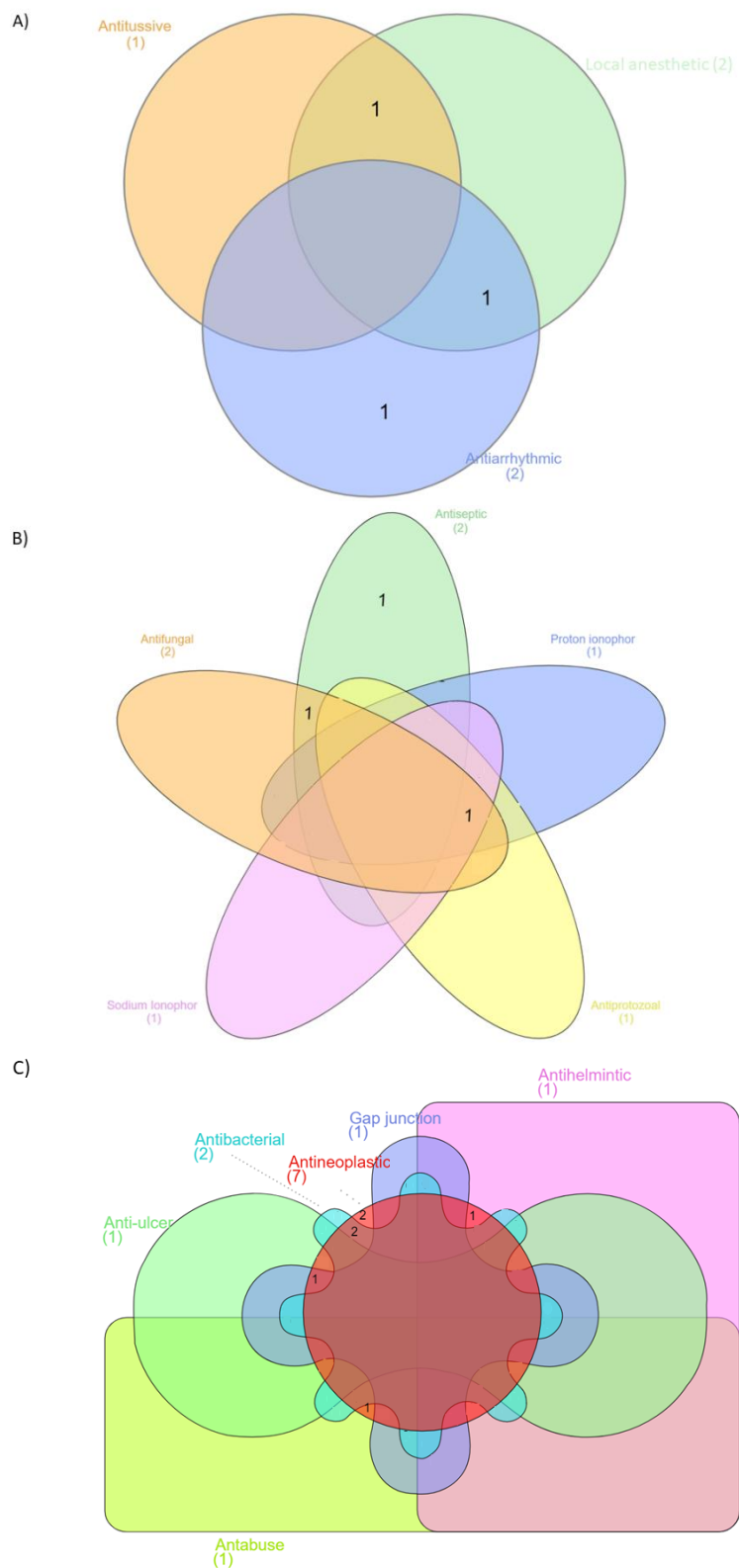


Figure A3. Interconnection between therapeutic effects of positive candidates represented in Venn diagrams. A) Relation between antitussive, local anesthetic and antiarrhythmic; B) Relation between antifungal, antiseptic, proton ionophores, sodium ionophores and antiprotozoal; C) Relation between antineoplastic, antibacterial, antihelminthic, anti-ulcer, gap junction and antabuse. Venn diagrams created with a bioinformatic tool (www.bioinformatics.psb.ugent.be/webtools/Venn/)

Positive Candidates									
Drug	Formula	Molecular Weight (g/mol)	Approved	Therapeutic effect	Target	Mechanism of action	Other reported relevant therapeutic effect	Expected BBB permeability*	Reference
Fulvestrant	C32H47F5O3S	606.80	FDA	Antineoplastic	Estrogen receptor	Binds competitively to ER	Neuroprotective against nerve degeneration	No	[249-252]
Atracurium besylate	C65H82N2O18S2	1243.51	FDA	Muscle relaxant	ACh receptor	Non-depolarizing blocker	Promote astroglia differentiation	Yes	[189, 200, 253]
Mexiletine hydrochloride	C11H18ClNO	215.72	FDA	Antiarrhythmic	Voltage-gated Na ⁺ channel	Inhibitory activity	Antimyotonic agent	Yes	[254-257]
			EMA	Local anaesthetic					
Flavoxate hydrochloride	C24H26ClNO4	427.93	FDA	Muscle relaxant	cAMP-dependent phosphodiesterase	Inhibitory activity	Parasympatholytic agent	Yes	[258-260]
Benzonatate	C30H53NO11	603.76	FDA	Antitussive	Voltage-gated Na ⁺ channel	Inhibitory activity	None	Yes	[261-264]
				Local anaesthetic					
Disulfiram	C10H20N2S4	296.54	FDA	Antabuse	Aldehyde dehydrogenase (ALDH)	Regulatory activity	Novel ADAM10 gene expression enhancer (target in Alzheimer's disease)	Yes	[188, 195, 196, 265]
				Antineoplastic	NPL4 receptor				
Danazol	C22H27NO2	337.47	FDA	Anti-estrogen	Estrogen receptor	Inhibitory activity	Reverse anemia and thrombocytopenia	Yes	[206, 208, 266, 267]
Troleandomycin	C41H67NO15	813.99	FDA	Antibacterial	50S ribosome subunit	Inhibitory activity	None	No	[268-270]
Hexetidine	C21H45N3	339.61	EMA	Antifungal	Enzyme	Inhibitory activity	None	Not found	[271, 272]
				Antiseptic					
Mitoxantrone dihydrochloride	C22H30Cl2N4O6	517.41	FDA	Antineoplastic	Topoisomerase II	Inhibitory activity	None	Not found	[273-275]
Dequalinium dichloride	C30H40Cl2N4	527.69	EMA	Antiseptic	Bacterial cell membrane	Increase permeability	None	Yes	[276-279]
					Protein involved in respiratory chain	Denaturation			
Tobramycin	C18H37N5O9	467.52	FDA	Antibacterial	Aminoglycoside receptor on the 30S ribosome subunit	Inhibitory activity	None	No	[280-282]

Flubendazole	C16H12FN3O3	313.28	EMA	Antineoplastic	Microtubule	Inhibitory activity	None	Not found	[283-286]
				Antihelmintic					
Bosentan	C27H29N5O6S	551.63	FDA	Vasodilator	Endothelial-receptor	Inhibitory activity	Reduces neuronal apoptosis	No	[287-290]
Pranlukast	C27H23N5O4	481.52	EMA	Antiasthmatic	Cysteinyl leukotriene receptor-1	Inhibitory activity	Neuroprotective effect; Ameliorating neuron degeneration	Inhibits/ Yes	[187, 202, 203, 291]
Monensin sodium salt	C36H61NaO11	629.87	EMA	Antifungal	Cellular and subcellular membranes	Disrupt the Na ⁺ /K ⁺ ion balance	None	Not found	[292, 293]
				Proton ionophores					
				Antiprotozoal					
				Sodium ionophores					
Dronedaron hydrochloride	C31H45ClN2O5S	593.23	FDA	Antiarrhythmic	Voltage-gated Na ⁺ channel	Inhibitory activity	None	Yes	[294-296]
					α - and β -adrenergic receptors				
					Voltage-gated K ⁺ channel				
					Ca ²⁺ channel				
Carbenoxolone disodium salt	C34H48Na2O7	614.74	EMA	Anti-ulcer	11-beta hydroxysteroid dehydrogenase	Inhibitory activity	FOXO3-inhibitory compound novel strategy against Alzheimer's disease and cognitive impairment	Yes	[297-299]
				Gap junction	PANX1				
				Antineoplastic	BIRC5/survivin				
Alexidine dihydrochloride	C26H58Cl2N10	581.73	EMA	Antibacterial	Bacterial cell membrane	Dysfunction	None	Not found	[300-302]
				Antineoplastic					
Novobiocin sodium salt	C31H35N2NaO11	634.62	FDA	Antibacterial	Topoisomerase II	Inhibitory activity	None	No	[303-305]
				Antineoplastic	BCRP-mediated topotecan transport				
Ethaverine hydrochloride	C24H30ClNO4	431.96	Taiwan	Antispastic	L-type Ca ²⁺ channel	Inhibitory activity	Monoamine oxidase (MAO) inhibition	Not found	[306-308]
Aprepitant	C23H21F7N4O3	524.44	FDA	Antiemetic	Neurokinin-1 (NK-1) receptor	Inhibitory activity	None	Yes	[191, 192, 309, 310]

Appendix B

Image analysis tool for evaluation of microtubule-stabilizing agents in ND7/23 differentiated cell model

In the following section, the image analysis process developed for quantification of microtubule-stabilization effect in ND7/23 differentiated cell model is explored. As described in Chapter V, section 2.1.3, cell evaluation includes pre-segmentation with ilastik program, image segmentation and analysis with CellProfiler™ software and final results analysis using Microsoft Excel.

B1. Image pre-segmentation using Ilastik

To improve the segmentation capacity of CellProfiler™ software, the images of nucleus and α -tubulin were pre-segmented (separately) with ilastik program. The objective of this initial step was to obtain images with better contrast to allow easier identification of primary objects (nucleus and tubulin).

In the ilastik program was used the pixel classification workflow, where are assigned labels to pixels (primary objective or background) due to user annotations and pixel features detected by the program. Briefly, after selecting a set of features the user paints accordingly with each label (class), creating a training set. Then, all the images are passed through the program to be pre-segmented with the training that the program was instructed as previously described (with a couple of images per condition). Ilastik output is an image with a probability map (Fig. B1) of each class, that further in CellProfiler™ can be selected in individual primary objects, the whiter the class the higher probability of corresponding to a primary object (nucleus or tubulin in each case).

B2. Image segmentation and analysis using CellProfiler™

For CellProfiler™ software analysis it was adapted a pipeline developed by Ferreira, H. which include the following modules [311]:

- i. Identity primary objects (Nuclei);
- ii. Identity secondary objects (Nuc expansion);
- iii. Identity tertiary objects (Collar)
- iv. Identity primary objects (Cell network);
- v. Measure size;
- vi. Mask objects (collar mask);
- vii. Mask objects (protrusions in collar);

- viii. Measure objects size;
- ix. Overlay outlines.

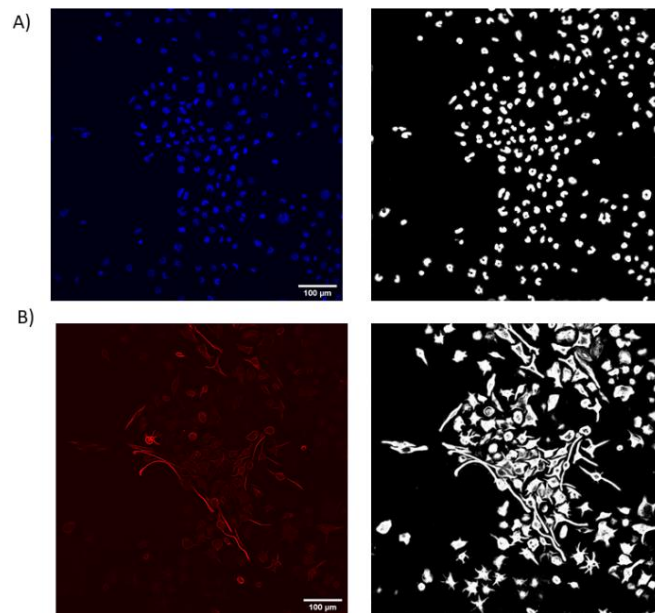


Figure B1. Representative images of before and after ilastik pre-segmentation. (A) IN Cell Analyzer 2000 nucleus image (right side) and corresponding Ilastik output (left side). (B) IN Cell Analyzer 2000 α -tubulin (right side) and ilastik output image (left side). Scale bars: 100 μ m.

B2.1. Identity primary objects (Nuclei)

The first module has the aim to identify individual nuclei as the primary object (region of interest for point of reference). For input are given the images obtained from Ilastik with DAPI channel (DAPI probabilities), and the objects identified are named “Nuclei”. Threshold method was manually determined by visual inspection and was set at an intensity of 0.6. Objects touching the border of the image are discarded and holes present in objects are automatically filled.

B2.2. Identity secondary objects (Nuc expansion)

The second module was developed to identify objects named “Nuc expansion”. Input images were DAPI probabilities and as reference were used the primary objects “Nuclei” (output images from first module).

The method used to identify secondary objects was the “Distance-N”. This method takes the edges of the primary objects “Nuclei” and expand them by 12 pixels (value determined manually), creating the secondary objects “Nuc expansion”. As well as in primary objects, secondary objects presenting holes were filled, but secondary objects touching the borders were considered.

B2.3. Identity tertiary objects (Collar)

The third module had the aim to create a “collar” around individual nucleus. The “collar” area is the peripheral space around, therefore, does not include the nucleus itself. Thus, tertiary objects

were created by subtracting the primary objects “Nuclei” to the respective secondary objects “Nuc expansion”.

B2.4. Identity primary objects (Cell network)

The aim of the fourth module was to again identify primary objects but having as input images from Ilastik with tubulin probabilities. These primary objects were named “Cell network”. In line with features adopted for the first module, it was set a manually threshold method of 0.6 of intensity. To obtain individualised cellular neurites, in this situation the holes were not filled.

B2.5. Measure object size

In this module the area of the primary objects “Cell network” was measured to be used in the final analysis of the results with Microsoft Excel.

B2.6. Mask objects (Collar mask)

In the sixth module the aim was to mask the primary objects “Cell network” to further only considered the tubulin processes (protrusions) located in the collar regions. To achieve this, the “Cell network” objects were selected to be masked and the tertiary objects “Collar” were used has a mask.

It is noteworthy that, in end of this module, two or more separated primary objects “Cell network” that are in the same collar are considered individual objects.

B2.7. Mask objects (Protrusions in collar mask)

To achieve the aim of calculate the total area occupied by protrusions inside an individual collar, those protrusions needed to be identified has the same object (which did not happen in the previously module has explained). Therefore, this module was developed to mask the tertiary objects “Collar” using the output of the previously model “Collar mask”.

B2.8. Measure object size

In this module different area objects (“Collar”, “Protrusions in collar”, and “Nuclei”) were measured to be used in the final analysis of the results with Microsoft Excel.

B2.9. Overlay outlines

In the final module, it was created images where are overlayed the outlines of the objects “Nuclei”, “Collar”, and “Collar mask” resorting to ilastik images from tubulin as background, showing the relation between these different objects (Fig. B2).

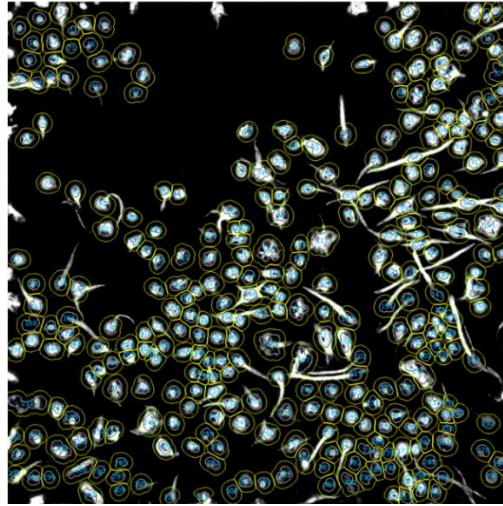


Figure B2. Representative image of final overlay of “Nuclei” (blue), “Collar” (yellow), and “Collar mask” (green) objects, using ilastik tubulin images as background.

B3. Microsoft Excel analysis

The final results analysis was performed following the same procedure described in the original paper by Boučanová and colleagues and by Ferreira, H. [218, 311]. Nevertheless, it was introduced some alterations since it was not possible to use the parameters provided in the paper since those corresponded to other IN Cell software and this cell analysis were used for ND7/23 cell line and not for oligodendrocytes has described. Therefore, samples of one independent experiment were analysed manually to determine the specific parameters for ND7/23 analysis, which were then applied throughout the remaining samples quantification.

Such as suggested by Boučanová *et al.*, it is necessary to “select well segmented cells” for a more accurate analysis of collar occupancy. The effect of microtubule-stabilization analysis was based on the concept of collar occupancy, i.e. ND7/23 cells were classified into different stages based the percentage of collar occupancy. Therefore, it is important to ensure that the collar area is proportional to the nucleus area, excluding cells whose collar area is limited by neighbouring cells, as it happens in highly confluent areas. To find the parameters that fulfil these conditions, it was manually analysed a plot of collar area vs nucleus area (Fig. B3).

Considering the plot from Fig. B3, each margin object was visually analysed until the limits that fulfil the conditions for collar occupancy analysis were set. Although the parameters were not very different from an independent experiment to another they can altered. Therefore, the establishment of parameters for proper collar occupancy analysis must be checked every independent experiment and adjusted if needed. For Fig. B3, the parameters established were maximums of (1235,2130) and minimums of (145,738), nucleus area and collar area, respectively (Table B1). All the objects with nucleus or collar areas outside these range were not considered. On the other side, cells inside parameters range were considered cell segmented and included for analysis.

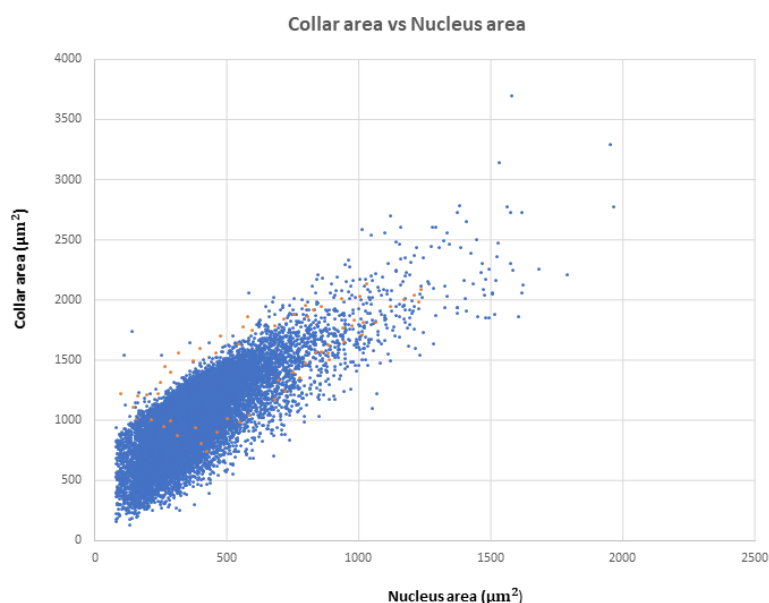


Figure B3. Plot of collar area vs nucleus area for each object in an analysed condition. Area values are in μm^2 . In orange dots are represented the limits in each outside of them the conditions of collar occupancy are not fulfil.

Finally, it was necessary to establish the different stages of ND7/23 cells (Table B1). The stages were determined by visual inspection and considering the percentage of collar occupancy ($\% \text{ collar occupancy} = \frac{\text{protusions in collar area}}{\text{Collar area}} \times 100$) and used for quantification of microtubule-stabilizing effect in ND7/23 cells.

Table B1. Parameters used in the ND7/23 microtubule-stabilization effect quantification analysis. All the values were determined by visual inspection.

Classification	Parameter	Value
Well segmented cells	Collar area	738-2130 μm^2
	Nucleus area	145-1235 μm^2
Collar occupancy stages	Stage I	0-22.0%
	Stage II	22.1-24.5%
	Stage III	>24.6%

Appendix C

Image analysis tool for evaluation of microtubule-stabilizing agents in primary cortical neurons model

The following section explores in detailed the image analysis process developed for quantification of microtubule-stabilization effect in mouse primary cortical neurons *in vitro* model. As described in Chapter V, section 2.2.1, the quantification process includes pre-segmentation with ilastik program, image segmentation and analysis with CellProfiler™ software and final results analysis using Microsoft Excel.

C1. Image pre-segmentation using Ilastik

To improve the segmentation tool of CellProfiler™ software, images of nucleus and β III-tubulin were first pre-segmented with ilastik program to allow a better image contrast and further easier identification of each object (nucleus and tubulin).

The procedure developed in ilastik program is the same already described in Appendix B, B1 section. Briefly, it was used the pixel classification workflow and it was created a training set with assigned labels (primary objects or background). All the images were run in the program creating an output of probability map for each input image.

C2. Image segmentation and analysis using CellProfiler™

For CellProfiler™ software analysis it was created a pipeline for skeleton analysis, which include the following modules:

- i. Identify primary objects (Nuclei);
- ii. Measure size and shape;
- iii. Filter objects (Filter Nuclei);
- iv. Identify secondary objects (Tub);
- v. Convert objects to image (Tub_NucExpanded);
- vi. Morphological skeleton (Skeleton_Tub);
- vii. Measure object skeleton;
- viii. Measure size and shape;
- ix. Overlay outlines.

C2.1. Identify primary objects (Nuclei)

The first module was design to identify individual nuclei as the primary object and, for that, it uses the DAPI probabilities, obtained from DAPI channel using Ilastik. The primary objects were named “Nuclei”.

“Nuclei” objects outside the diameter range of 6-50 pixel units and touching the border were discarded to eliminate debris. Threshold method was manually set to an intensity of 0.3 and holes were filled after applying the threshold.

C2.2. Measure size and shape

In this module the Zernick features of the “Nuclei” primary objects were measured.

C2.3. Filter objects

The third module had the aim to identify live and dead cells. Therefore, “Nuclei” primary objects are filtered by their size.

Anilkumar *et al.* used an image analysis method to distinguish live and apoptotic cells by their nucleus characteristics, translating smaller and denser nuclei has apoptotic neurons [222]. The same method was applied in this module, “Nuclei” objects were filtered by their maximum radius. The “Nuclei” objects with less than 8.6 pixel units (value selected by visual inspection) were discarded and counted has dead in the final excel results and the positive filtered primary objects were called “Filter Nuclei”.

C2.4. Identify Secondary Objects

The aim of this module was to identify objects named “Tub”. Input images were tubulin probabilities and the objects “Filter Nuclei”.

The method used to identify the secondary objects was by the “Propagation”. This method finds dividing lines between different objects by using a combination of the distance to the closest “Filter Nuclei” and the gradients of intensity, creating the secondary objects “Tub”, which means that it starts from the “Filter nuclei” and spreads by the input tubulin probabilities and stops immediately after finding a gap (to identify destabilization/stabilization phenotypes), creating objects “Tub”.

A manual threshold of 0.5 and a regular factor (controls the balance between the two factors considered in the propagation method, nearest primary object distance and intensity and) of 0.05 were set. As well as in primary objects, holes present in secondary objects were automatically filled, but objects touching the borders were considered.

C2.5. Convert objects to image (Tub_NucExpanded)

The fifth module was developed to convert “Tub” secondary objects into image in a binary (black & white) colour format. The output images were named “Tub_NucExpanded”.

C.2.6. Morphological skeleton

This module converts the input image “Tub_NucExpanded” to the output image “Skeleton_Tub”, by creating a morphological skeleton (Fig. C1 (B)).

C.2.7. Measure object skeleton

This module was developed to measure the number of total branches (with a branch being a pixel with more than two neighbours) and endpoints (a pixel with only one neighbour) in the skeletonized image “Skeleton_Tub” from the previous model.

C.2.8. Measure object size and shape

The aim of this module was to calculate the Zernick features of the “Filter Nuclei” objects.

C.2.9. Overlay outlines

In the final module, it was created images where are overlaid the images of the objects “Filter Nuclei” and “Tub” resorting to tubulin ilastik and “Skeleton_Tub” images, respectively (Fig. C1).

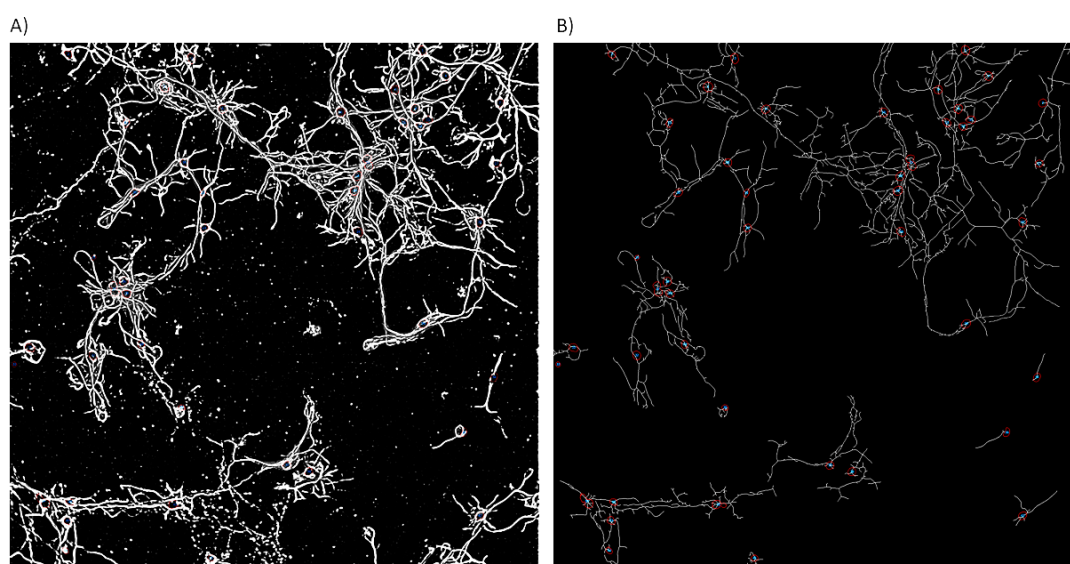


Figure C1. Representative image of final overlay of: A) “Filter Nuclei” (red) objects, using ilastik tubulin images as background; and B) “Tub” objects, using “Skeleton_Tub” images as background.

C3. Microsoft Excel analysis

The output from the CellProfiler™ software pipeline for skeleton analysis includes a variety of information regarding all nucleus, the filtered nucleus, and the morphological skeleton, including total number of objects, Zernick features, and total skeleton length.

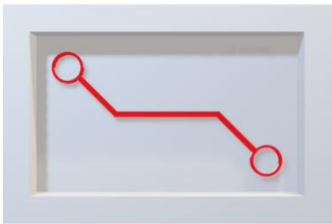
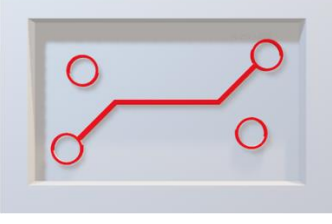
To proceed with the analysis for the evaluation of neuron microtubule-stabilization/destabilization effect it was used the total skeleton length measured to calculate the mean skeleton length of each identify cell. Moreover, the % cell death was also calculated for each condition using the number of total primary objects “Nuclei” first identify and then the number of objects “Nuclei filter” each give us the number of live cells.

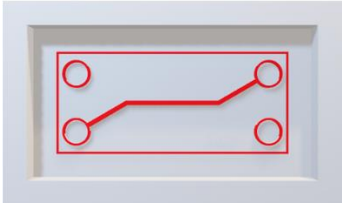
Appendix D

3D printed BBB-on-a-chip templates

In the following section are presented the different 3D templates produced for optimization of the microfluidic devices that composed the BBB-on-a-chip (Table D1), template “BBB_astro” (mould of the upper microfluidic) has 7 different versions and the template “BBB_endo” (mould of the lower microfluidic) has 4 different versions. First versions were developed in the SCAD software and the final versions were developed in the SolidWorks software, a CAD 3D software more advanced. An example of the code developed in SCAD software for the design of the 3D templates is also shown ahead.

Table D1. Presentation of all the versions and what designs alterations occurred during optimization of 3D templates of BBB-on-a-chip device microfluidics.

Template	Version	Design alterations	3D design
BBB_astro	1	First template	
	2	Reverse the channels for a correct cross position with the lower microfluidic when assembling the BBB-on-a-chip. Addition of more 2 inlets precisely aligned with inlets from the “BBB_endo” to allow direct reach from above for the channels of bottom microfluidic	
	3	Bring closer the 2 inlets previously added, misaligning them slightly from the lower microfluidic, but allowing the liquid to contact directly with the feeding channel enable flow from the inlet to the channel	
	4	Angle between feeding channels and central channel was curved to allow a better 3D printing	

	5	Alteration of external shape of the template so that the microfluidic can have the same shape such as coverslips available in the lab.	
	6	Translation for solid works software	
	7	Addition of a guidance line around the channels and inlets to allow consistent manually cut of microfluidics for proper dimensions accordingly with the available coverslips	
BBB_endo	1	First template	
	2	Alteration of external shape of the template so that the microfluidic can have the same shape such as coverslips available in the lab.	
	3	Translation for SolidWorks software. The inlets were filled as there was no need to punch them since these channels are accessed by the upper microfluidic	
	4	Addition of a guidance line around the channels and inlets to allow consistent manually cut of microfluidics for proper dimensions accordingly with the available coverslips	

Code line developed in the SCAD software for the design of template “BBB_endo” version 1:

```
//BBB endothelial
```

```
//Function rounded cubes
```

```

// More information: https://danielupshaw.com/openscad-rounded-corners/

// Set to 0.01 for higher definition curves (renders slower)

$fs = 0.15;

module roundedcube(size = [1, 1, 1], center = false, radius = 0.5, apply_to = "all") {

    // If single value, convert to [x, y, z] vector

    size = (size[0] == undef) ? [size, size, size] : size;

    translate_min = radius;

    translate_xmax = size[0] - radius;

    translate_ymax = size[1] - radius;

    translate_zmax = size[2] - radius;

    diameter = radius * 2;

    obj_translate = (center == false) ?

        [0, 0, 0] : [

            -(size[0] / 2),

            -(size[1] / 2),

            -(size[2] / 2)

        ];

    translate(v = obj_translate) {

        hull() {

            for (translate_x = [translate_min, translate_xmax]) {

                x_at = (translate_x == translate_min) ? "min" : "max";

                for (translate_y = [translate_min, translate_ymax]) {

                    y_at = (translate_y == translate_min) ? "min" : "max";

                    for (translate_z = [translate_min, translate_zmax]) {

                        z_at = (translate_z == translate_min) ? "min" : "max";

                        translate(v = [translate_x, translate_y, translate_z])

                        if (

                            (apply_to == "all") ||

                            (apply_to == "xmin" && x_at == "min") ||

                            (apply_to == "xmax" && x_at == "max") ||

                            (apply_to == "ymin" && y_at == "min") ||

                            (apply_to == "ymax" && y_at == "max") ||

```



```

//inlet R

translate([18.87,8.87,0])

difference(){

cylinder(0.2,3,3,$fn=100,true);

cylinder(0.2,2.5,2.5,$fn=100,true);

}

//inlet R

translate([-18.87,-8.87,0])

difference(){

cylinder(0.2,3,3,$fn=100,true);

cylinder(0.2,2.5,2.5,$fn=100,true);

}

}

//base

difference(){

cube([60,40,10],true);

translate([0,0,2.5])

cube([50,30,5],true);

}

//nome molde

mirror(v=[1,0,0])

translate([15,12,0])

linear_extrude(0.5)

text("BBB Endo v1", size=1);

```

Appendix E

Coating evaluation on promoting non-wetting surfaces hydrophilicity: contact angle measurements

Table E1 includes the different conditions evaluated by measurement of contact angle (using pendant drop method) and the results obtained. This information complements in detail data presented in Chapter VI, Section 3.2.

Table E1. Contact angle measurements performed by pendant drop method. Present values correspond to measurements made with 0.5 μ L water drop, n=1 independent experiment with three contact angle measurements *per* condition. All measurements are in degrees ($^{\circ}$).

BBB-on-a-chip composed	Coating	Measured contact angle	
Bottom microfluidic (Microfluidic for endothelial cells seeding)	None (PDMS material)	96.5 $^{\circ}$	
		91.7 $^{\circ}$	
		92.7 $^{\circ}$	
	100 μ g/mL collagen (4 $^{\circ}$ C, overnight)	98.6 $^{\circ}$	
		92.9 $^{\circ}$	
		93.1 $^{\circ}$	
	2 mg/mL PDA (RT, 24h)	30.2 $^{\circ}$	
		17.4 $^{\circ}$	
		9.7 $^{\circ}$	
	2 mg/mL PDA (RT, 48h)	21.8 $^{\circ}$	
		23.5 $^{\circ}$	
		35.1 $^{\circ}$	
	2 mg/mL PDA (RT, 24h) + 100 μ g/mL collagen (4 $^{\circ}$ C, overnight)	$\approx 0^{\circ}$ (No possible to measure due to extreme hydrophilicity)	
PET porous membrane	None (PET material)	70.5 $^{\circ}$	
		70.2 $^{\circ}$	
		68.0 $^{\circ}$	
	100 μ g/mL PDL (37 $^{\circ}$ C, overnight)	70.1 $^{\circ}$	
		70.0 $^{\circ}$	
		55.4 $^{\circ}$	
	Plasma treatment	0 minutes after treatment	30 minutes after treatment
		22.2 $^{\circ}$	43.6 $^{\circ}$
		21.3 $^{\circ}$	46.8 $^{\circ}$
		44.7 $^{\circ}$	42.6 $^{\circ}$
	2 mg/mL PDA (RT, 24h)	30.4 $^{\circ}$	
		25.0 $^{\circ}$	
		27.2 $^{\circ}$	
	2 mg/mL PDA (RT, 48h)	27.2 $^{\circ}$	
		21.4 $^{\circ}$	
		21.1 $^{\circ}$	
	2 mg/mL PDA (RT, 24h) + 100 μ g/mL collagen (4 $^{\circ}$ C, overnight)	33.5 $^{\circ}$	
		30.3 $^{\circ}$	
		34.8 $^{\circ}$	

Top microfluidic (Microfluidic for astrocytes seeding)	2 mg/mL PDA (RT, 24h) + 100 µg/mL PDL (38°C, overnight)	34.1°
		13.9°
		34.2°
	None (PDMS material)	97.6°
		106.3°
		95.3°
	100 µg/mL PDL (37°C, overnight)	88.9°
		105.4°
		110.6°
	2 mg/mL PDA (RT, 24h)	87.5°
		90.6°
		75.5°
	2 mg/mL PDA (RT, 24h) + 100 µg/mL PDL (38°C, overnight)	≈0° (No possible to measure due to extreme hydrophilicity)