The importance of Cyclophilins and Mps1 in cancer

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Sonia Simón Serrano

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Abstract: Hepatic fibrosis is a pathological response to chronic liver injuries such as chronic alcohol consumption, non-alcoholic fatty liver disease (NAFLD) or chronic viral infections. Currently no antifibrotic compound has been approved for liver fibrosis and if the cause of the chronic injury is not solved, fibrosis can progress to cirrhosis. Currently the only treatment for liver cirrhosis is liver transplantation. Cirrhosis is associated with increased risk in the development of hepatocellular carcinoma (HCC), the most common primary liver cancer.

Cyclophilins are intracellular proteins with the capacity to catalyse the cis/trans isomerization of the peptide bonds at the proline residues facilitating protein folding and conformational changes affecting the function of the targeted proteins. Cyclophilin overexpression is a common event in fibrotic tissues playing a key role in different stages of the fibrotic process, including inflammation, hepatocyte death, and activation of the hepatic stellate cells leading to increased collagen production. We investigated the antifibrotic effect of NV556, a novel potent sanglifehrin-based cyclophilin inhibitor *in vitro* and *in vivo*. NV556 decreased liver fibrosis in two animal models of Non-alcoholic steatohepatitis (NASH), STAM and methionine-choline-deficient (MCD) mice. NV556 treatment in an *in vitro* 3D human liver Extracellular Matrix (ECM) cultured with a TGFβ1-activated human hepatic stellate cell line led to decreased collagen production.

HCC is usually diagnosed at a late stage with few accepted treatments and limited efficacy due to side effects and resistance to the treatment. Overexpression of cyclophilins has been observed in HCC increasing proliferation, metastasis and promoting chemoresistance. Treatment with the novel cyclophilin inhibitor NV651, presented a potent antiproliferative effect in HCC cell lines via cell cycle perturbations arresting cells in the mitotic phase. In addition, NV651 decreased tumour growth in xenograft-mouse model. We could also confirm the safety in normal cells and good oral bioavailability. Several pathways involved in cell cycle and DNA repair were affected upon NV651 treatment. The combination of NV651 and cisplatin, a DNA damage reagent, resulted in a synergistic effect in cell viability, and a significant increase on cell death in HCC cell lines. This combination caused a disturbance in the cell cycle and a decrease in the capacity of the cell to repair interstrand crosslinks.

Targeting the cell cycle has been proposed as a therapeutic strategy in cancer treatment with special interest in the mitotic phase. The mitotic kinase monopolar spindle 1 (Mps1) is a key regulator of the Spindle assembly checkpoint, which ensures the correct chromosome segregation. We could confirm the increased polyploidy upon Mps1 inhibition in neuroblastoma cell lines and PDX model, leading to mitotic catastrophe activating the caspase-dependent mitochondrial apoptotic pathway. In addition, Mps1 inhibition decreased tumour growth in a xenograft mouse model.

In summary, cyclophilin inhibition in liver fibrosis and HCC could be used as a potential therapeutic strategy, individually or in combination. Inhibition of Mps1 led to a potent cytotoxic effect indicating its potential use as a treatment against neuroblastoma.

Key words
Cyclophilins, liver, fibrosis, HCC, cell cycle, Mps1 and mitosis

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"Lo difícil no es ganar dinero sin más... Lo difícil es ganarlo haciendo algo a lo que valga la pena dedicarle la vida."

— *Carlos Ruiz Zafón, La sombra del viento*

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List of papers

This thesis is based on the following papers:

I. *Evaluation of NV556, a Novel Cyclophilin Inhibitor, as a Potential Antifibrotic Compound for Liver Fibrosis*

S. Simon Serrano, A. Grönberg, L. Longato, K. Rombouts, J. Kuo, M. Gregory, S. Moss, E. Elmér, G. Mazza, P. Gallay, M. Pinzani, M. J. Hansson, and R. Massoumi.

Cells 8, no. 11 (Nov 8 2019).

II. *Novel Cyclophilin Inhibitor Decreases Cell Proliferation and Tumor Growth in Models of Hepatocellular Carcinoma*

S. Simon Serrano*, M. Tavecchio*, A. Grönberg, W. Sime, M. Jemaa, S. Moss, M. A. Gregory, P. Gallay, E. Elmér, M. J. Hansson, and R. Massoumi.

Cancers (Basel) 13, no. 12 (Jun 18 2021).

III. *Synergistic effects of sanglifehrin-based cyclophilin inhibitor, NV651, with cisplatin in hepatocellular carcinoma*

S. Simon Serrano, J. Malik, M. Tavecchio, A. Grönberg, E. Elmér, C. Kifagi, M. J. Hansson, and R. Massoumi.

Manuscript.

IV. *Inhibition of mitotic kinase Mps1 promotes cell death in neuroblastoma*

S. Simon Serrano, W. Sime, Y. Abassi, R. Daams, R. Massoumi, and M. Jemaa.

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*Equal contribution

Abbreviations

ACTA2	Actin Alpha 2
AASLD	American Association for the Study of Liver Diseases
ABC	ATP-binding cassette
ALT	Alanine aminotransferase
ARID1A	AT-rich interactive domain-containing protein 1A
ARID2	AT-Rich Interaction Domain 2
AST	Aspartate aminotransferase
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
ATR	Ataxia Telangiectasia and Rad-3 related
BA	Biliary Atresia
Bcl-2	B-cell lymphoma 2
BCLC	Barcelona Clinic Liver Classification
BDL	Bile duct ligation
BRCA1	Breast cancer type 1
CAT	Catalase
CCl4	Carbon tetrachloride
CCND1	Cyclin D1
CD147	Cluster of differentiation 147
Cdc25A	Cell division cycle 25 homolog A
CDDP	Cisplatin
CDK	Cyclin-dependent kinases
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
Chk	Checkpoint kinase

CLD	Cyclophilin-like domain
COL1A1	Collagen Type I Alpha 1 Chain
CsA	Cyclosporin A
CTNNB1	Catenin Beta 1
CypA	Cyclophilin A
CypB	Cyclophilin B
CypD	Cyclophilin D
DDR	DNA damage response
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
EMT	Endothelial-mesenchymal transition
ER	Endoplasmic reticulum
ERCC-1	Excision repair cross complementation group 1
ERK	Extracellular signal-regulated kinase
GEPIA	Gene Expression Profiling Interactive Analysis
GG-NER	Global-genome nucleotide excision repair
GPx	Glutathione peroxidase
GSH	Glutathione
H2AX	H2A histone family member X
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HFD	High fat diet
HIF	Hypoxia-inducible factor
HRE	Hypoxia-response element
HSC	Hepatic stellate cell
IL	Interleukin
iPS	induced Pluripotent stem cell

LOX	Lysyl-oxidase
MAPK	Mitogen-activated protein kinase
MCD	Methionine- and Choline-Deficient Diet
MDM2	Mouse double minute 2 homolog
MEC	Minimum effective concentration
MMP	Matrix metalloproteinase
MPS1	Monopolar spindle 1
MPTP	Mitochondrial Permeability transition pore
NAC	N-acetyl cysteine
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFAT	Nuclear factor of activated T cells
NIM811	N-methyl-4-isoleucine cyclosporine
NSCLC	Non-small cell lung carcinoma
PARP	Poly (ADP-ribose) polymerase
PDGF	Platelet-derived growth factor
PDX	Patient-derived xenograft
PEI	Percutaneous ethanol injection
PI3K	Phosphoinositide 3-kinases
PPI	Peptidyl-prolyl isomerase
PPIL3	Peptidyl-Prolyl Isomerase-Like 3
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcoma
RB	Retinoblastoma
RFA	Radiofrequency ablation
ROS	Reactive oxygen species
RPTEC	Renal proximal tubule epithelial cells
SAC	Spindle Assembly Checkpoint
SfA	Sanglifehrin A

SOD	Superoxide dismutase
STAT3	Signal transducer and activator of transcription 3
STZ	Streptozotocin
TACE	Transarterial chemoembolization
TCGA	The Cancer Genome Atlas
TC-NER	Transcription-coupled nucleotide excision repair
TERT	Telomerase Reverse Transcriptase
TGF-B1	Transforming growth factor-b1
TGFBR	Transforming growth factor-beta receptor
TIMP	Tissue inhibitors of metalloproteinases
TP53	Tumor protein P53
TRR	Thioredoxin
USP4	Ubiquitin-specific protease 4
VEGFR	Vascular endothelial growth factor receptor
VLDL	Very low-density lipoprotein
XPC	Xeroderma pigmentosum C
α-SMA	α-smooth muscle actin

Abstract

Hepatic fibrosis is a pathological response to chronic liver injuries such as chronic alcohol consumption, non-alcoholic fatty liver disease (NAFLD) or chronic viral infections including hepatitis B and C. Previous research has presented the reversibility of fibrosis when the cause of the chronic injury is solved, shining a light on the importance of developing effective antifibrotic compounds.

Cyclophilins (Cyp) are intracellular proteins with the capacity to catalyse the cis/trans isomerization of the peptide bonds at the proline residues facilitating protein folding and conformational changes affecting the function of the targeted proteins. Cyclophilin overexpression is a common event in fibrotic tissues playing a key role in different stages of the fibrotic process, including inflammation, hepatocyte death, and activation of the hepatic stellate cells (HSC) leading to increased collagen production. In this study, we investigated the antifibrotic effects of NV556, a novel potent sanglifehrin-based cyclophilin inhibitor *in vitro* and *in vivo*.

The potential effect of cyclophilin inhibition by NV556 was evaluated in two well-established animal models of NASH, STAM and methionine-choline-deficient (MCD) mice; as well as in an *in vitro* 3D human liver ECM culture of LX2 cells, a human hepatic stellate cell line. We demonstrate that NV556 decreased liver fibrosis in both STAM and MCD *in vivo* models and decreased collagen production in TGFβ1-activated hepatic stellate cells *in vitro*. Taken together, these results present NV556 as a potential candidate for the treatment of liver fibrosis.

Liver cirrhosis has been associated to an increased risk in the development of hepatocellular carcinoma (HCC), the most common primary liver cancer. Cisplatin is a DNA damage reagent that can be administered to the patient diagnosed at an intermediate stage. On the other hand, HCC is usually diagnosed in its late stage with few accepted treatments and limited efficacy due to side effects and resistance to the treatment. Therefore, with these limited treatment options, there is a need for novel effective treatments against HCC.

Although cyclophilins are found in all types of cells and organisms, overexpression of these proteins has been observed in HCC increasing proliferation, metastasis and promoting chemoresistance. In HCC, we evaluated the effect of cyclophilin inhibition by using NV651, a novel and potent cyclophilin inhibitor. NV651 treatment in HCC cell lines resulted in inhibited proliferation *in vitro* via cell cycle perturbations and arrest of the cells in the mitotic phase. In addition, tumour growth

was inhibited in a xenograft-mouse model upon cyclophilin inhibition. We could also confirm the safety in normal cells and good oral bioavailability, presenting NV651 as a potential candidate for HCC treatment. By evaluating changes in the transcriptome by NV651 treatment in HCC cell lines, we could observe several affected pathways related to cell cycle and DNA repair. We investigated the potential synergistic effect between NV651 and treatments against HCC. NV651 and cisplatin presented a synergistic effect on cell viability and a significant increase on cell death in HCC cell lines. Combination of NV651 and cisplatin caused a disturbance in the cell cycle in comparison to single treatment of cisplatin and a decrease in the capacity of the cell to repair crosslinks. Based on these results, we believe that the combination of cisplatin and NV651 could provide a valuable addition to the treatment arsenal in HCC.

Targeting cell cycle has been proposed as a therapeutic strategy in cancer treatment with special interest in the mitotic phase. The mitotic kinase monopolar spindle 1 (Mps1) is a key regulator of the Spindle assembly checkpoint, which ensures the correct chromosome segregation. We could confirm the increased polyploidy upon Mps1 inhibition in neuroblastoma cell lines and PDX model, leading to mitotic catastrophe activating the caspase-dependent mitochondrial apoptotic pathway. In addition, Mps1 inhibition decreased tumour growth in a xenograft mouse model, indicating that the inhibition of Mps1 could be used as a potential treatment for neuroblastoma.

Popular summary

Liver fibrosis appears when a healthy liver becomes scarred impeding the correct function of the liver. Liver fibrosis can be caused by persisting liver infections, alcohol, or fatty liver due to obesity. If the cause is not removed, the liver can become more scarred, this is known as cirrhotic liver. Patients with a cirrhotic liver present a higher risk of developing hepatocellular carcinoma (HCC), the most common primary liver cancer and the sixth most common type of cancer. It is commonly diagnosed at a late stage and the median survival comprises between 6 and 20 months. When the tumour is detected at an advanced stage, the alternative is palliative treatment, where only the symptoms of the disease can be treated with limited options available.

Cyclophilins are proteins found inside the cells able to guide other proteins to have the correct structure in a short period of time, an activity that is necessary for the correct function of the cells. Although cyclophilins are found in all the cells of all known organisms, some types of human cyclophilins are present in higher levels during fibrosis and cancer.

Cyclophilins are important for the different events leading to the appearance of liver fibrosis. We have studied the effect of inhibiting cyclophilins in mice that develop liver fibrosis and in a cell type important for the development of fibrosis by producing products such as collagen facilitating the scarring of the liver. We could see an overall decrease in liver fibrosis when cyclophilins were inhibited in mice and a decrease in the production of collagen, presenting the inhibition of cyclophilins as an interesting treatment for liver fibrosis.

In HCC, these increased levels have been correlated to a decreased survival in patients and an increased resistance of cancer cells to several treatments against HCC. The individual effect of cyclophilin inhibition or in combination with treatments against HCC in cancer cell lines was evaluated. We could confirm that our cyclophilin inhibitor was not toxic and was able to decrease the proliferation of cancer cells by altering the cell division. We also observed a reduction in the tumour growth in mice.

As cyclophilins have been linked to treatment resistance, we evaluated whether inhibiting the function of cyclophilins can increase the cell death of cancer cells with chemoagents used for HCC. If the effect of this combination is higher than the sum of the individual effects, it would result in the so-called synergistic effect,

presenting it as a potential effective combination therapy for HCC. We could confirm a synergistic effect on cell viability and increased apoptosis, a type of cell death, with our cyclophilin inhibitor and cisplatin, a chemoagent used for HCC that damages the DNA. We believe this combination presents synergistic effect because cisplatin induces DNA damage and the inhibition of cyclophilins decrease the capacity of the cells to repair this damaged DNA. As the cells are not able to repair the DNA damage this will lead to cell death.

The cell cycle is the process that the cells go through to divide and duplicate. Mitosis is a phase where one cell divides into two identical cells. This phase has been of extreme interest for the treatment against cancer. We have evaluated the effect of inhibiting the activity of the mitotic kinase monopolar spindle 1 (Mps1) in neuroblastoma. Mps1 is an important protein that regulates the correct division of the cell. Neuroblastoma is one of the most common cancer types in children that forms in certain types of the nerve tissue. We could show that by inhibiting Mps1, cells were not able to divide properly, and this led to cell death. We also confirmed the decrease in tumour growth in mice when Mps1 was inhibited, presenting Mps1 inhibition as an interesting treatment against neuroblastoma.

Resumen popular

Fibrosis hepática puede aparecer cuando un hígado sano se cicatriza impidiendo la correcta función de este órgano. La fibrosis de hígado puede ser causada por infecciones crónicas, alcoholismo o hígado graso causado por obesidad. Si la causa no es eliminada, el hígado puede aumentar su cicatrización hasta llegar a la fase de cirrosis. Los pacientes con un hígado cirrótico presentan una probabilidad más alta de desarrollar carcinoma hepatocelular o CHC, el cáncer primario de hígado más común y el sexto cáncer más común. CHC es normalmente diagnosticado en una etapa avanzada con una media de supervivencia entre 6 y 20 meses. Cuando el tumor es detectado en una etapa avanzada, las opciones terapéuticas son escasas y la única alternativa es tratamiento paliativo, donde solo los síntomas de la enfermedad pueden ser tratados.

Las ciclofilinas son proteínas que se encuentran en el interior de las células, capaces de guiar a otras proteínas para obtener una estructura correcta en un período corto de tiempo. Esta función es necesaria para el correcto funcionamiento de las células. Aunque las ciclofilinas se encuentran en todas las células de todos los organismos conocidos, algunos tipos de ciclofilinas humanas están presentes en niveles más altos durante el desarrollo y progreso de fibrosis y CHC.

Las ciclofilinas son importantes para diferentes eventos que promueven la aparición de fibrosis hepática. En esta tesis hemos estudiado el efecto producido al inhibir la actividad de las ciclofilinas en ratones con fibrosis hepática y en una población celular importante para el desarrollo de la fibrosis ya que pueden sintetizar productos necesarios para la cicatrización del hígado como el colágeno. La inhibición de las ciclofilinas dio lugar a una disminución general de fibrosis hepática y una disminución en la producción de colágeno. Estos resultados presentan la inhibición de ciclofilinas como un tratamiento interesante para la fibrosis hepática.

En CHC, el aumento de la expresión de ciclofilinas se ha correlacionado con una menor supervivencia en los pacientes y una mayor resistencia de las células cancerígenas a varios tratamientos para CHC. Hemos evaluado el efecto individual de la inhibición de ciclofilinas o en combinación con tratamientos utilizados para CHC en líneas celulares cancerosas. Estos resultados han demostrado que nuestro inhibidor de ciclofilinas no presentaba ningún indicio de toxicidad y podía reducir la proliferación de células cancerígenas al alterar la división celular. También pudimos confirmar una reducción en el crecimiento tumoral en ratones.

Como las ciclofilinas se han relacionado con la resistencia al tratamiento contra CHC, hemos evaluado si nuestro inhibidor puede reducir la viabilidad de células cancerígenas con compuestos utilizados para la quimioterapia contra CHC. Si el resultado de esta combinación es mayor que la suma de los efectos individuales, esto resultaría en el llamado efecto sinérgico, presentando esta combinación como una terapia potencialmente efectiva para el tratamiento de CHC. Los resultados de estos experimentos han indicado un efecto sinérgico en la reducción de la viabilidad combinando nuestro inhibidor con cisplatino, un compuesto utilizado para la quimioterapia en CHC que daña el ADN. Esta combinación resultó en un incremento significativo de células apoptóticas. Hemos hipotetizado que este incremento en muerte celular puede estar relacionado al incremento de daño al ADN debido al tratamiento de cisplatino y una reducción en la capacidad de reparación del ADN por la inhibición de las ciclofilinas. Al no poder reparar el ADN, las células activan la muerte por apoptosis.

El ciclo celular es el proceso necesario por el que pasan las células para dividirse. La mitosis es una fase en la que una célula se divide en dos células idénticas. Esta fase ha sido de gran interés para el tratamiento contra el cáncer. Hemos evaluado el efecto de inhibir la actividad de *monopolar spindle 1* (Mps1) en neuroblastoma. Mps1 es una proteína necesaria para la correcta división de la célula. Neuroblastoma es uno de los tipos de cáncer pediátrico más comunes que se forma en ciertos tipos de tejido nervioso. Hemos demostrado que al inhibir Mps1, las células no podían dividirse correctamente y esto ha dado lugar a su muerte celular. También pudimos confirmar la disminución del crecimiento tumoral en ratones debido a la inhibición de Mps1. Con estos resultados, creemos que la inhibición de Mps1 podría ser un tratamiento interesante contra neuroblastoma.

Populärvetenskaplig sammanfattning

Leverfibros uppträder när en frisk lever bildar ärrvävnad som hindrar leverns normala funktion. Leverfibros kan orsakas av infektioner, alkoholkonsumtion eller fetma. En långt gående leverfibros utan behandling kan utvecklas till det farliga stadiet som kallas för skrumplever eller levercirros. Patienter med levercirros har en mycket högre risk att utveckla levercancer som är den sjätte vanligaste typen av cancer. Levercancer diagnostiseras vanligtvis i ett sent skede på grund av att patienterna är symptomfria med medianöverlevnad mellan 6–20 månader. Det finns begränsade alternativ för behandlingsmetoder när levercancer upptäcks i ett framskridet stadium.

Cyklofiliner är viktiga proteiner som styr strukturen av andra proteiner i cellen som är nödvändiga för att cellerna ska fungera korrekt. Vissa typer av mänskliga cyklofiliner förekommer i högre nivåer i specifika sjukdomstillstånd som fibros och cancer. Vi har studerat effekten av att hämma Cyklofiliner hos möss som utvecklar leverfibros. Behandling av djuren med cyklofilinhämmare ledde till en total minskning av leverfibros på grund av minskade mängd av fibrosmaterialet kollagen hos behandlade djuren. Baserad på dessa resultat kan hämning av cyklofiliner betraktas som en potentiell behandlingsstrategi för patienter som lider av leverfibros.

Höga nivåer av cyklofiliner i levercancer har korrelerats till en minskad överlevnad hos patienter och en ökad resistens hos cancerceller mot flera anti-cancerbehandlingar. När vi studerade den individuella effekten av cyklofilinhämning i kombination med andra behandlingsmetoder visade det sig att cyklofilinhämmare påverkar cancercellernas proliferation genom att förändra celldelningen. Dessutom kunde en synergistisk effekt när det gäller ökade celldöd i celler som behandlades med cisplatin och cyklofilin hämmare påvisas. Cisplatin är en slags cellgift som används för behandling av levercancerpatienter. Vi tror att kombinationen av cisplatin som inducerar DNA-skada och cyklofilinhämning som minskar kapaciteten hos cancercellerna att reparera skadat DNA kan förklara synergin med ökad cancercelldöd. Behandling av djur med cyklofilinhämmare minskade också tumörtillväxten hos möss.

Cellcykeln är den process som cellerna går igenom för att kunna dela sig och duplicera. Mitos är en fas där en cell delar sig i två identiska celler. Vi har utvärderat effekten av att hämma aktiviteten hos den mitotiska kinasmonopolära spindeln 1

(Mps1) i cancersjukdomen neuroblastom. Neuroblastom är en av de vanligaste cancertyperna hos barn som bildas i vissa typer av nervvävnad. Vår studie kunde visa att hämning av Mps1 påverkar celldelningen och driver cancercellerna till celldöd. Vi kunde också bekräfta minskningen av tumörtillväxt hos möss när Mps1 aktiviteten blockerades, vilket tyder på att Mps1-hämning kan vara en alternativ behandlingsmetod för neuroblastompatienter.

Background

The liver

The liver is a key organ for the maintenance of physiological homeostasis situated under the diaphragm and protected by the lower ribs. The liver is the first organ to receive the absorbed nutrients and other substances entering through the gastrointestinal tract acting as a gate-keeper [1] with functions involving metabolism, detoxification of chemicals, macronutrient metabolism, blood volume regulation and immune regulation among others [2].

The liver is organized into macroscopic divisions known as lobes differentiated by anatomy and blood supply while microscopically, the liver structures in hexagonal shapes, known as a hepatic lobule (Figure 1). The hepatic lobule is limited by the portal triad in the vertices formed by a hepatic artery, portal vein and bile ducts, delivering bile produced by the hepatocytes needed for digestion and absorption of lipids (Figure 1) [1].

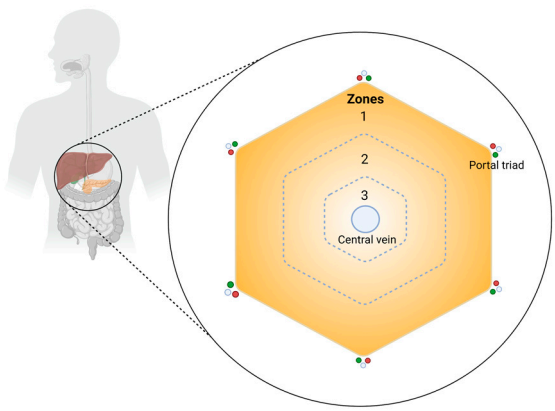

Figure 1. Structure of the hepatic lobule, decreasing gradient is correlated to oxygen concentration. Adapted from "Liver, Pancreas, and Gallbladder with Callout (Layout)", by BioRender, December 2020, https://app.biorender.com/biorender-templates/t-61746a04a4981000a6d10500-liver-pancreas-and-gallbladder-with-callout-layout Copyright 2021 by BioRender.

The blood supply of the liver has two different origins. The hepatic artery provides 25% of the blood volume and is highly oxygenated [3, 4] while the portal vein, provides a highly nutritious blood contributing to around 75% of the blood volume proceeding from the gastrointestinal tract, spleen, transporting senescent red blood cells, gallbladder and pancreas [1, 2, 4-6]. Blood from the portal vein and hepatic artery will perfuse the liver through capillaries known as sinusoids, where blood is then mixed while transported to the middle of the lobule to the central vein forming the hepatic vein [1, 2]. During this transport, cells along the lobule will utilize this blood, create metabolites, and waste products, creating a gradient of oxygen and nutrients, where different functions will also be performed depicted in the form of zones [2, 6].

Macronutrient metabolism is one of the most important functions of the liver including carbohydrate metabolism. The liver is a key organ for the maintenance of glucose levels in blood and is able to use substrates originated from nutrient absorption from the gastrointestinal tract [1]. The functions in the liver are controlled by the glucagon to insulin ratio, during feeding, glucose uptake increases glycolysis for energy production or glycogen production for storage [1, 2]. The liver can store glucose derived from glycogen or gluconeogenesis from amino acids, lactate, pyruvate, and glycerol coming from other organs. When in fasting state, insulin levels will decrease and glucagon increase, causing the liver to release glucose to other parts of the body that demand energy [2].

The liver is also involved in fat metabolism playing a key role in the absorption, uptake, synthesis, storage, and secretion of lipids. The liver produces most of the lipoproteins, cholesterol, and phospholipids for the body. A high percentage of the cholesterol produced in the liver is used for the formation of bile acids [1].

The liver is also involved in protein metabolism such as interconversion of amino acids [1]. In addition, the liver can handle toxic components such as nitrogen due to deamination during amino acid metabolism resulting in production of urea (ureagenesis) that will be later on secreted by the kidneys [2, 6]. The liver secretes a high level of proteins to the bloodstream such as albumin, important for the maintenance of the blood volume, or factors for blood coagulation [2, 6].

The liver takes care of removing haemoglobin from senescent red blood cells from the spleen that come to the liver via the portal vein and are phagocyted by the Kupffer cells [2, 6]. The liver is the first defense for drugs orally administrated including alcohol, as they will be absorbed in the small intestine and transported to the liver via the portal vein [6].

Overfeeding can lead to hepatic insulin resistance where insulin cannot reduce the release of glucose, increasing blood glucose but can still stimulate lipogenesis, contributing to NAFLD development [2].

The liver is formed by several cell types.

The hepatocytes are the primary epithelial cells found in the liver. This cell population constitutes most of the liver volume and comprises 80% of the total cells. Hepatocytes are polarized and specialized epithelial cells with a basolateral membrane towards the perisinusoidal space of Disse, located between the basolateral membrane of the hepatocyte and the sinusoid and containing the blood plasma. In this space, the microvilli of the hepatocytes can be found, facilitating the absorption of proteins and nutrients in the plasma. In the apical membrane the adjacent cells form a continuous network that drains in the bile ductules known as the canaliculi [1, 6].

Cholangiocytes or biliary epithelial cells are found in the lumen of the bile ducts and comprise the second biggest epithelial population in the liver.

Hepatic stellate cells (HSC) are non-parenchymal liver cells with stellate morphology found in the perisinusoidal space of Disse [7, 8]. HSC can be found in an active or quiescent state. During the quiescent state, these cells can store intracellularly vitamin A in large lipid droplets. Due to their contractility, HSC can regulate the hepatic blood flow and portal venous pressure [9, 10]. Activation of HSC due to liver damage will increase proliferation while losing their storage capacity. During the active state, HSC can modify the ECM [2, 11], an important activity that will be discussed in the next sections.

In the liver, a macrophage population known as Kupffer cells play an immunological role including the cleansing from potential bacteria absorbed from the gut [1] and can present pro- and anti-inflammatory functions depending on the environmental factors. They are normally situated close to the pores of the sinusoidal endothelial cells, and they are in contact with the portal blood flow. Other functions include the recycling of senescent red bloods cells.

Finally, the liver sinusoidal endothelial cells, forming pores through fenestrations keeping a selectable barrier enable the exchange of small proteins and molecules in the liver between the circulating blood and hepatocytes [2, 6].

Pathology of the liver

Due to an increasing percentage of the population presenting overweight and obesity, several health consequences have been linked to the increase comorbidities such as non-alcoholic fatty liver disease (NAFLD) and liver cancer. NAFLD has the highest prevalence in South America (31%), Middle East (32%), Asia (27%) and USA (24%) with the lowest percentage in Africa (14%) while in Europe the median prevalence is between 23 and 26% [12].

Non-alcoholic steatohepatitis (NASH) is a component of the metabolic syndrome characterized by obesity, type II diabetes mellitus, and dyslipidaemia [11]. NASH is the severe progressive form of NAFLD and is characterised by steatosis with macro or microvesicular lipid accumulation in at least 5% of hepatocytes. Steatosis is followed by hepatocyte injury presenting hepatocellular ballooning, enlarged hepatocytes with a clumped cytoplasm that could have been originated from residual fat droplets, specific from steatohepatitis [13]. In NASH the initial injury is often produced in zone 3, near the hepatic vein due to lower levels of oxygen and higher levels of Reactive oxygen species (ROS)[14]. This is followed by chronic inflammation or hepatitis, that can lead to liver fibrosis, excessive wound healing where connective tissue replaces the normal parenchyma (Figure 2) [15, 16]. Patients presenting NASH will rarely experience symptoms until reaching the cirrhotic stage, this happens in an interval between 15 and 20 years, although symptoms such as chronic fatigue, malaise, and discomfort have been reported [11, 17].

The cirrhotic stage is difficult to revert, and no standard treatment has been approved yet, so liver transplantation is the only curative option. At the cirrhotic stage, excessive deposition in the extracellular matrix causes changes in the anatomy and structure of the liver with formation of fibrous scar and decreased blood supply impeding the correct functions of the liver [18, 19]. During cirrhosis, regenerating nodules can emerge facilitating the transformation of normal hepatocytes to dysplastic hepatocytes, and finally HCC due to accumulation of genetic changes through cell division [20-23]. Therefore, cirrhosis has been associated to an increased risk in the development of hepatocellular carcinoma (HCC)(Figure 2) [24], the most common primary liver cancer [24, 25].

Hepatic fibrosis

In addition to NASH, hepatic fibrosis is a pathological response to other chronic liver injuries such as chronic viral infections including HBV and HCV and chronic alcohol consumption. Liver fibrosis is characterised by excessive wound healing where connective tissue replaces the normal parenchyma due to continuous extracellular matrix remodelling [7, 26] distorting the liver architecture. These changes in the quantity and composition of the ECM are caused by increased synthesis and decreased degradation [27], regulated by the ratio of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [11].

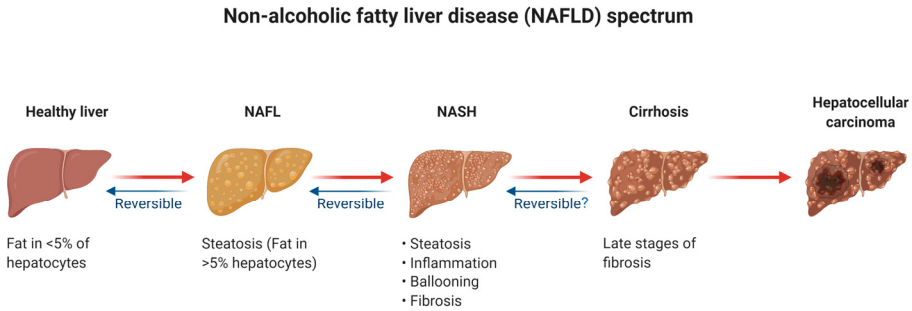

Figure 2. Development of HCC from healthy liver. Adapted from "Non-Alcoholic Fatty Liver Disease (NAFLD) Spectrum", by BioRender, July 2020, https://app.biorender.com/biorender-templates/t-5f7b2888f7396700abd2cf23-non-alcoholic-fatty-liver-disease-nafld-spectrum Copyright 2021 by BioRender.

Hepatic stellate cells

Under physiological conditions, hepatic stellate cells (HSC) are found in the space of Disse in a quiescent non-proliferative state and with capacity to store vitamin A as previously mentioned. Upon liver injury, HSC will transdifferentiate to active myofibroblasts [28]. Activated HSC lose their capacity to store vitamin A and become the main collagen and ECM producing cells in the development of liver fibrosis, leading to tissue remodelling and repair [7, 29]. Activated HSC also present enhanced proliferation, fibrogenic capacity with increased accumulation of fibrillar collagens, especially collagen I and III and production of chemotactic substances to recruit inflammatory cells and other HSCs [7, 9, 30-32]. Their modified cytoskeleton promotes migration, adhesion and contractility [7]. Collagen production is regulated at several levels including transcriptionally and post-transcriptionally [33]. Activated HSC express several markers including collagen type I, α-smooth muscle actin (α-SMA) (gene ACTA2), transforming growth factor-β1(TGFB1), MMPs and TIMPs that facilitate the fibrotic development [34].

Several cell populations such as hepatocytes, Kupffer cells and neutrophils can interact with HSCs and induce their activation. Apoptosis of hepatocytes will directly and indirectly activate the fibrogenic capacity of the myofibroblasts in the liver. Inflammatory cells can activate HSC for collagen secretion with cytokines such as IL-17, a pro-inflammatory and profibrogenic cytokine [35, 36].

Two of the most known fibrogenic factors are TGF-β, the most important cytokine for activation and fibrogenic stimulation of HSC and PDGF, the most important cytokine for HSC proliferation [7, 37, 38].

TGF-β can be secreted by several cell types such as Kupffer cells, the main producers of TGF-β, HSC [39] or HBV infected hepatocytes [40]. TGF-β1 can bind to TGFBRII, that phosphorylates TGFBRI. This activates the Smad cascade, by phosphorylating Smad2/3 inducing the formation of a complex together with Smad4 translocating to the nucleus and increasing the expression of pro-fibrotic factors such as PAI-1, TIMPs, TGFB1 and MMPs and the transcription of collagen I and III. Smad also induces Smad7 that will negatively regulate the TGF- β pathway [28, 41-43].

A previous study [40] has presented a hypothesis where TGF-β pathway causes a positive feedback loop between TGF-β1 and CD147 (Basigin or extracellular matrix metalloproteinase inducer (EMMPRIN)), needed to activate HSC. CD147 is expressed in low levels in quiescent HSC but TGF-β1 treatment can increase the expression of CD147 by augmenting the phosphorylation levels of Smad2 and 3 and translocating Smad4 to the nucleus binding to the CD147 promoter and directly increasing its expression. This leads to increased phospho-levels of ERK1/2, increased Sp1 protein levels and activation of HSC. The activation of HSC causes an increased expression of COL1A1, TGFB1 and ACTA2 (α-SMA). The role of CD147 was confirmed with additional experiments where increased migration and contraction of HSC indicated the importance in the transdifferentation of HSC into active myofibroblasts. Co-localization of CD147 and α-SMA positive cells in the perisinusoidal space was identified in the fibrotic liver in CCl_4-model and in human cirrhotic liver biopsies, identifying HSC with both factors. In LX2 cells, overexpression of CD147 increased the expression of ACTA2, TIMP1, COL1A1, MMP-13, MMP-2 and TGFB1, also leading to increased CD147 expression. Transfected CD147 reported an increased growth while inhibition of CD147 increased apoptosis in HSC. In addition, liver fibrosis was ameliorated upon CD147 blockage in the CCl_4-model decreasing collagen levels, TIMP-1 and ACTA2 expression [10]. Increased apoptosis indicated an interesting approach for liver fibrosis reversibility.

Treatments

Currently no antifibrotic drug have been approved for liver fibrosis in humans and the best approach is to remove the causative agent of the liver injury [44]. Reversibility of liver fibrosis has been observed when the cause of chronic injury is removed both *in vivo* and in patients [39, 45-50]. During resolution, HSC can have different fates, undergo apoptosis or inactivation [11, 46]. Inactive or senescent HSC will be more sensitive to fibrogenic stimuli than quiescent cells [46, 49].

Different approaches have been proposed including anti-inflammatory drugs to diminish the inflammatory stage or antioxidants that protect hepatocytes and decrease fibrosis [51]. Direct approaches such as blocking the TGF-β signalling pathway have also been proposed preventing scar formation *in vivo* [37]. Inhibition of collagen production or increase its degradation [52] have also been proposed.

Obeticholic acid is a Farsenoid X receptor agonist with the ability to increase insulin sensitivity and decrease steatosis, hepatocellular ballooning, inflammation, and fibrosis [53, 54]. Obeticholic acid is currently in clinical trial phase III [44].

Telmisartan is an angiotensin II receptor antagonist and peroxisome proliferator-activated receptor-γ agonist able to reduce fibrosis and lobular inflammation [55].

Hepatocellular carcinoma

Liver cancer was classified as the sixth most common type of cancer with an incidence of 905677 new cases and the third most common type of cancer-related death with 830180 deaths worldwide in 2020 [56]. Two types of primary liver cancer can be found, cholangiocarcinoma (<10%) and hepatocellular carcinoma or HCC (>90% of the cases). HCC is most prevalent in Southeast Asia and in sub-Saharan Africa [25, 57].

HCC can be caused by chronic viral infections including HBV or HCV, aflatoxin exposure, chronic alcohol consumption and non-alcoholic fatty liver disease (NAFLD) [58]. Worldwide, chronic HBV and HCV are the most common cause, where in Asia (mainly China) and Africa up to 90% of HCC cases can be originated from chronic HBV [59]. In the US and Europe chronic HCV is the most common HCC cause while aflatoxin exposure is the main cause in localized regions of Africa [20, 59].

On the other hand, cases in United States and other developed countries are rising [60] due to the increase in obesity and type II diabetes that positively correlate with the increase in HCC cases [61]. Specifically HCC cases linked to NASH have been rising due to the increased percentage of people presenting obesity and diabetes worldwide, currently between 2.4% and 12.8% of NASH-related cirrhosis will develop HCC [62].

Several mechanisms are involved in the development of HCC including tumour microenvironment, necroinflammation, oxidative stress, hypoxia, and DNA damage among others [20]. HCC development is a multi-step process linked to chromosomal aberrations and altered gene expression. From a molecular point of view, the most commonly mutated genes are involved in telomere maintenance (*TERT*), cell cycle (*TP53*, *CCND1* and *CDKN2A*), transcription (*CTNNB1*) and chromatin remodelling (*ARID1A* and *ARID2*), but many other cellular functions are deranged due to additional mutations [20, 63].

HCC is commonly diagnosed at an advanced stage with a median survival of several months [57]. At the curative stage, one of the main problems is recurrence of HCC with more than 70% of the cases at 5 years [64]. Another additional problem is that HCC is commonly found in a dysfunctional liver decreasing the options of using curative approaches.

HCC patients can present cancer and liver dysfunction-related symptoms including weight loss, pain in the upper right quadrant, ascites, jaundice (yellow pigmentation of the skin and eyes), fever, nausea and vomiting and lack of energy among others [65]. Unfortunately, at later stages, metastasis is still a major contributor for treatment failure and bad prognosis [66]. HCC tends to metastasize, mainly to lung, lymph nodes, bones, and adrenal glands [67], causing a decrease in patients' life quality. Therefore, new drugs with novel mechanism of action are urgently needed.

Staging

Although there is no official staging system The Barcelona Clinic Liver Cancer (BCLC) staging system has been endorsed by The American Association for the Study of Liver Diseases (AASLD). The BCLC determines cancer stage and patient prognosis based on tumor burden, severity of liver disease, and the patient's performance status [68]. This staging system is commonly used in clinical as well as being used to test effectivity for HCC treatments for several clinical trials [69]. A schematic view can be found in Figure 3.

At very early (stage 0) and early stage (A) patients present one solitary lesion or up to 3 nodules of ≤3 cm each with no macrovascular invasion or extrahepatic spread [68]. At this stage, patients can receive curative treatment [70] including resection, transplantation, or radiofrequency ablation (RFA).

Resection is classically the first-line curative surgical option for HCC. Resection can be chosen as the surgical treatment in patients presenting only one isolated tumour, no vascular invasion and preserved liver function [71, 72]. In addition, it is important to evaluate whether the remaining liver volume can function properly without increasing morbidity and mortality [73, 74]. Unfortunately, only around 20% of patients can be candidates of liver resection due to location, size or number of tumours or hepatic dysfunction [75].

Liver transplantation is the best option for HCC patients presenting a cirrhotic liver [76]. The feasibility of an HCC patient for liver transplant follows the Milan Criteria: single HCC tumors <5cm or three tumors ≤3cm with no macrovascular invasion on imaging, by this criterion the 4-years survival rate reaches 75% [77]. The main limitation of liver transplantation is the low number of livers available and the long waiting period.

Patients diagnosed at an early stage presenting co-morbidities and unable to have surgical approaches are normally treated with Percutaneous local ablation including radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI). Similar survival of patients has been observed with RFA in a tumor ≤3cm in comparison to resection [78].

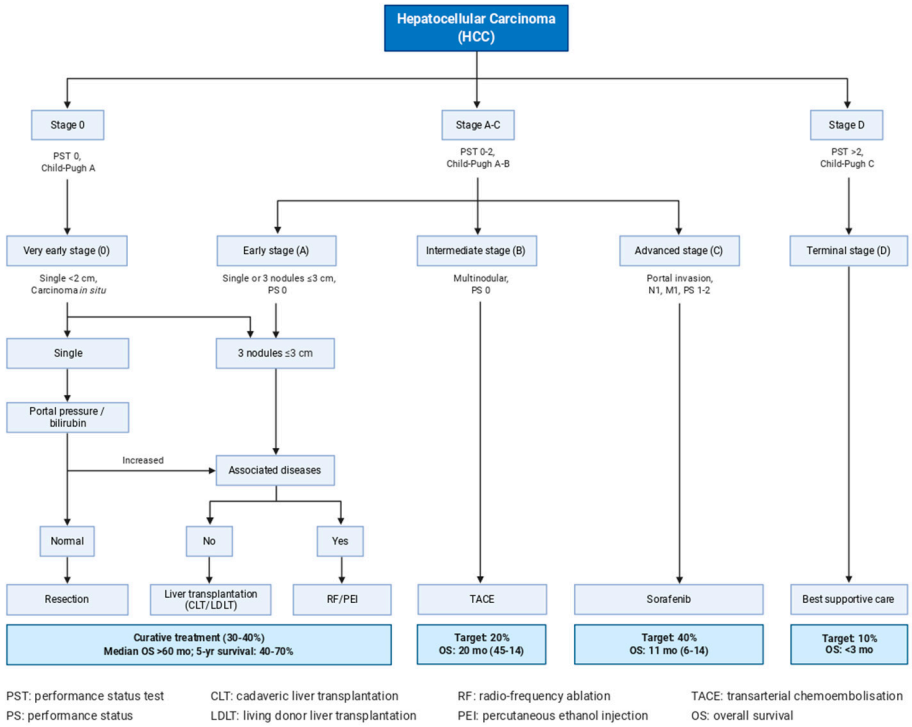

Figure 3. Barcelona Clinic Liver Cancer Classification (BCLC) staging and system strategy. Adapted from "Barcelona Clinic Liver Cancer (BCLC) Staging System", by BioRender, July 2020, https://app.biorender.com/biorender-templates/t-5efa6d1fbc0c9f00ab59e095-barcelona-clinic-liver-cancer-bclc-staging-system Copyright 2021 by BioRender.

Patients diagnosed at an intermediate stage (B) present multiple large tumours without vascular invasion or extrahepatic invasion [68]. Optimal patients for Transarterial chemoembolization (TACE) present preserved liver function, no vascular invasion or extrahepatic spread [68]. In addition, TACE has been used to reduce tumors in HCC and be able to pass the Milan criteria [79, 80]. TACE presents a dual activity by occluding the hepatic arteries with agents such as gelatin sponge [81]. The embolization of the hepatic artery using a catheter has a dual function through occlusion of the hepatic arteries blocking the blood flow and therefore inducing hypoxia-induced death and by accumulating chemoagents in the tumour area [82]. TACE survival reaches 63% in the second year for an unresectable HCC tumour [83]. The success of TACE depends on the complete obstruction of tumor vascularization [81]. TACE will be stopped if there is tumor progression or loss of liver function [68]. During TACE, chemoagents such as mitomycin, doxorubicin and cisplatin are widely used before the embolization [84]. DNA damaging reagents are the most common therapy for human cancers although side

effects include unspecific targeting of normal cells specifically high proliferative cells limiting its efficacy [85].

Cisplatin is a DNA damage reagent that targets both nuclear and mitochondrial DNA also increasing the levels of reactive oxygen species (ROS) [86]. Cisplatin can induce several types of DNA damage such as inter and intrastrand crosslinks DNA adducts that can block DNA replication and transcription [87]. Unfortunately, treatments with chemoagents are often limited due to chemoresistance. Several types of resistance can appear such as reduced uptake leading to decreased intracellular accumulation, intracellular inactivation of the compounds, increased levels of glutathione or increased DNA repair [87-90]. In addition, as TACE can lead to hypoxia, this can increase HIF-1α activation promoting chemoresistance and antioxidant activity in HCC [91].

At advanced stage, patients present extrahepatic invasion, macroscopic vascular invasion and/or mild cancer related symptoms [70]. HCC is usually diagnosed in a late stage due to the lack of symptoms in patients and until recently the only treatment available at this stage was sorafenib.

Sorafenib is a systemic therapy given to patients in advanced HCC with preserved liver function and unable to have any of the options described above or have not been successful with the previous treatments [92, 93]. Sorafenib is a tyrosine kinase inhibitor targeting Raf isoforms, VEGFR and PDGFR, affecting several functions such as cancer cell proliferation, apoptosis and angiogenesis [94, 95]. In addition, sorafenib can block the ERK pathway that can lead to extrinsic and intrinsic apoptosis at advanced HCC [96]. Several side effects have been reported upon treatment including vomiting, arterial hypertension, skin reactions and weight loss [97, 98]. Side effects often lead to reduction of dosage or termination of the treatment [99]. Sorafenib can prolong the life expectancy up to 3 months [98], before resistance develops [100].

Recently, other tyrosine kinase inhibitors have been accepted for first line systemic therapy including Lenvatinib [101] and for second line systemic therapy: regorafenib [102], cabozantinib [103] and ramucirumab [104]. On the other hand, none of these novel compounds present a significant increase in life expectancy in comparison to sorafenib [101-104].

At the end stage (D) patients present poor liver function and/or obvious cancer-related symptoms. This stage is untreatable and only supportive care can be given. Unfortunately patients present poor prognosis at this stage [105].

Liver fibrosis and HCC animal models

STAM model

The STAM model was initially introduced by Fujii, Shibazaki [106] for the evaluation of the link between diabetes and HCC. NASH is induced in mice with chemical and dietary interventions by exposure to low dose of streptozotocin (STZ) two days after birth and high fat diet (HFD). STZ disrupts pancreatic β cells, inducing diabetes, and promotes adiposity in the liver. At 4 weeks of age, mice are fed ad libitum with HFD, this increases fat deposition and lipogenesis in the liver as well as fatty acid oxidation increasing oxidative stress [107] and causing hepatocellular injury [108, 109]. Inflammatory macrophages are recruited to create inflammatory foci and to phagocytose the lipid droplets [110]. That leads to a continuous activation of HSC in Disse's space causing histological changes from fatty liver to the appearance of tumours. NASH is usually developed around week 8 and HCC at 16 to 20 weeks. At week 8, fatty liver can be observed with moderate inflammation and ballooning. Between week 8 and 12 pericellular fibrosis takes place close to the central veins (zone 3). Collagen type I and III are upregulated in this model before the fibrotic phase as seen in other fibrotic models [111].

The MCD model

The Methionine and Choline-Deficient Diet (MCD) model has been widely used and is based on the administration ad libitum of a diet high in sucrose (40%) and moderate fat (10%) with lack of methionine and choline, necessary for hepatic mitochondrial β-oxidation and very low-density lipoprotein synthesis (VLDL). This will induce an impairment in triglyceride clearance inducing a lipid accumulation in the liver. In addition the impaired β-oxidation increases oxidative stress [112]. The benefit of this model includes the phenotypical traits from human NASH in a short time period [113]. The diet induces steatosis and oxidative stress and cytokine changes contributing to liver injury with rapid development of steatohepatitis (between week 5 and 8 of diet). At week 10 of diet, increased plasma ALT levels can be observed and pericellular fibrosis mainly in zone 3 similar to humans. On the other hand, mice fed with MCD diet will present weight loss due to hypermetabolic state [114] and decreased plasma triglycerides and cholesterol as well as a decreased liver/body ratio. The MCD model presents decreased blood glucose levels and does not present insulin resistance opposite to humans [113].

BDL model

Bile duct ligation (BDL) is an experimental model of cholestasis, causing blockage of bile secretion. The obstruction causes an accumulation of bile acids inducing a liver injury due to toxicity in hepatocytes and cholangiocytes leading to necrosis and apoptosis followed by fibrosis and cirrhosis [115] .

CCl_4-model

Hepatocytes express cytochrome P450 important for the conversion of xenobiotics, drugs, and toxins to inactivate metabolites that can be excreted with bile. Usually if it is in high concentrations, intermediates of drugs can lead to a toxic intermediate [1]. The CCl_4-model is induced by injecting CCl_4 that is metabolised by P450. This leads to the production of a toxic intermediate inducing damage in DNA, proteins and lipids causing altered lipid metabolism leading to steatosis, hepatocyte death, inflammation, fibrosis, cirrhosis and finally development of HCC [116].

Xenograft animal models

For the evaluation of tumour growth in animal models, one of the most common options are the injection of cancer cells either orthotopically where cancer cells are injected in the corresponding tissue of the animal model or subcutaneously (SC) xenografted. New techniques have appeared for the evaluation of tumour growth such as In vivo imaging system (IVIS). This instrument can perform non-invasive whole body bioluminescence (BLI) [117]. BLI has minimal background signal in comparison to fluorescence (high signal/background ratio), increasing its sensitivity and detecting a low number of cells [118, 119]. Cancer cell lines such as HEPG2 can be transduced with a viral vector containing luciferin activated with the ubiquitin C promoter (Figure 4)[120]. The luciferase transgene is fused to GFP where the latter can be used for the sorting of transduced cells. Some of the inconveniencies are the potential interference of hair, therefore it is recommended the use of nude mice for improvement of 2D bioluminescence. Position is also important and how deep the signal is for optimal exposure [117]. Orthotopic and SC xenografted mice need to be immunodeficient to decrease any potential immune reaction. Therefore nude mice that contain a genetic mutation presenting an absent or deteriorated thymus decreasing immune response can be optimal for this type of studies [121]. IVIS is a system able to analyse tumour growth at an early stage, therefore treatments can be started before the tumour is visible by eye [119]. On the other hand, necrotic tumours can present reduced bioluminescent signal so other methods such as caliper measurement would be needed to confirm a reduction in the tumour growth.

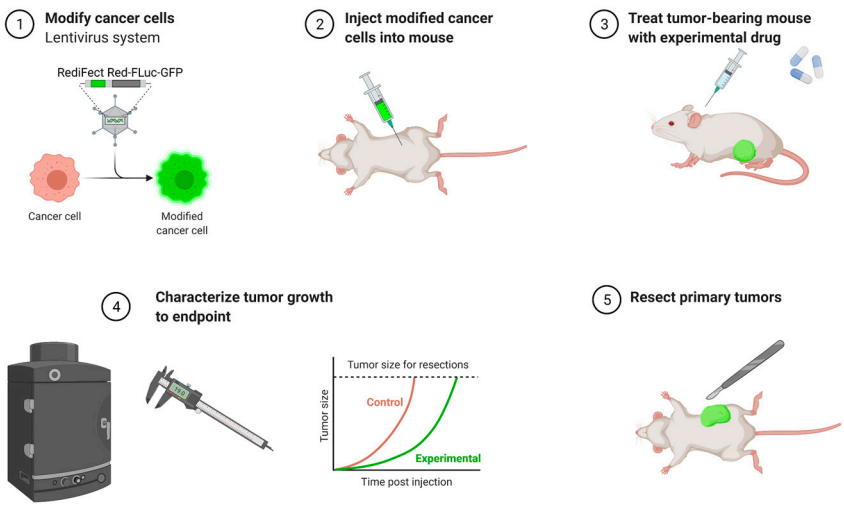

Figure 4. Schematic view of IVIS and treatment evaluation. From cell transduction to resection of the tumor. Adapted from "Mouse Model of Cancer Metastasis", by BioRender, July 2021, https://app.biorender.com/biorender-templates/t-5fb300900b3b2e00a6403e4a-mouse-model-of-cancer-metastasis Copyright 2021 by BioRender.

The Cell cycle

During the cell cycle a sequence of phases are divided in interphase and mitosis where interphase can be further divided in G_0, G_1, S and G_2. During the G_1 phase cells are growing in size and preparing for the DNA replication. During S phase, replication of the DNA takes place and in G_2 cells will then prepare for the mitosis by synthetizing organelles and proteins and increase further in size. Finally, mitosis will take place where a parental cell will lead to two identical daughter cells. The cells have a regulatory system where entries into the different phases are controlled by cyclin-dependent kinases (Cdk) forming complexes with a regulatory cyclin. On the other hand, alterations in the regulation/checkpoints of the cell cycle in cancer cells are often seen [122].

DDR and checkpoints

Currently most treatments against cancer induce DNA damage or target cell division or chromosome segregation. By doing so, cell cycle checkpoints are normally activated. This can include DNA damage reagents such as cisplatin interfering in DNA replication and leading to activation of checkpoints. In this way, cells can have more time to repair the DNA damage for a correct cell division resulting in two identical daughter cells. If a correct DNA damage repair is not completed, cells can activate mechanisms of cell death [85].The activation of the DNA damage response or DDR coordinates cell cycle progression, DNA replication and repair and apoptosis [123]. DDR presents different stages including DNA damage recognition, incision of the damaged area, synthesis of the new DNA segment and ligation [87].

The DDR is comprised of several factors that detect and lead to the activation of several checkpoints including G_1, S and G_2/M in relation to DNA damage [124]. Depending on the type of DNA damage detected and the cell cycle phase, different factors will be activated. The Ataxia Telangiectasia Mutated (ATM) kinase can activate the G_1/S checkpoint blocking the transition to S phase when double strand breaks in the DNA are detected through activation of the Check-point kinase 2 or Chk2. Chk2 inhibits the phosphatase Cdc25 impeding the activation of cyclin E/ Cdk2 complex [125].

The Ataxia Telangiectasia and Rad3 related (ATR) kinase activates Chk1 due to stalled replication forks in the S phase, activated Chk1 degrades Cdc25 and blocks cells in the intra S phase [85]. When DNA damage is detected in G_2/M, ATR can inhibit the entry to mitosis by blocking Cyclin B/Cyclin B-dependent kinase 1 (Cdk1) activation [126] with Wee1, a kinase blocking the entry into mitosis of cells with incomplete DNA replication or damaged DNA, so cells have time to repair the DNA [127]. In addition Chk1 can also inhibit Cdc25C [85]. Chk1 inhibitors have been tested in combination with cisplatin, this impedes the cells from repairing the DNA, progressing to mitosis and leading to mitotic catastrophe [85]. ATR inhibitors have also been used in combination with genotoxic agents, this combination increased the sensitivity drugs such as cisplatin or doxorubicin in breast and ovarian carcinoma [128].

Cancer cell lines can normally present mutations in key factors of the cell cycle checkpoint activation. P53 encoded by TP53 is important for the cell cycle, DNA repair, senescence and apoptosis and is frequently inactivated in cancer [129, 130].

Mitotic catastrophe

If cells with DNA damage do pass to the mitotic phase, they will go into permanent arrest or cell death through mitotic catastrophe. Cells undergoing mitotic catastrophe will die during mitosis or the daughter cells during G_1 phase will undergo cell death or senescence. Although still under debate, cells undergoing

mitotic catastrophe might die through different mechanisms including apoptosis, necrosis or autophagy or a combination of the different mechanisms [131].

When the intrinsic apoptotic pathway is activated, a downregulation of the mitochondrial membrane potential is observed normally by the opening of the mitochondrial permeability transition pore (MPTP) situated in the mitochondria. Calcium is a well-known activator of the MPTP, and its intracellular concentration can be increased due to aberrant cell cycle that can be caused by DNA damage [132]. Other factors such as increased ROS levels can also induce the opening of the MPTP. The MPTP is formed by VDAC (voltage-dependent anion channel, a channel protein found in the outer mitochondrial membrane and presenting direct exchange of metabolites), ANT (adenine nucleotide translocator-1, in the inner mitochondrial membrane regulating ATP and ADP transport between mitochondria and cytoplasm) and cyclophilin D (CypD) that regulates the opening of the pore [133]. The opening of the MPTP can release pro-apoptotic proteins such as Cytochrome C to the cytoplasm, causing apoptosome formation, caspase activation and DNA fragmentation [134, 135].

The Spindle assembly checkpoint

Targeting cell cycle has been proposed as a therapeutic strategy in cancer treatment with special interest in the mitotic phase [136]. During mitosis the spindle assembly checkpoint or SAC controls the proper cell division through the correct attachment of the chromosomes to the microtubule spindle apparatus through the kinetochores, centromeric structures that interact with spindle microtubules [137]. SAC is activated through the mitotic checkpoint complex or MCC that is upregulated due to tensionless kinetochores. MCC is formed by BubR1, Bub3 and Mad2 [85]. During metaphase the SAC will be inactivated when the chromosomes have been correctly placed on the metaphase plate and chromosome segregation will take place [85]. On the other hand, progression to anaphase will be delayed until proper completion of the mitotic spindle assembly is completed [85]. This is regulated by SAC inhibiting the anaphase-promoting complex/cyclosome (APC/C) and blocking the degradation of Cyclin B and securing (inhibitor of anaphase) until proper attachment of the chromosomes to the microtubules takes place [138].

Microtubule destabilizing agents can bind to β-tubulin altering the mitotic spindle assembly by either stabilizing the microtubules such as taxanes including paclitaxel or docetaxel or by preventing the polymerization of the microtubule such as vinca-alkaloids including vincristine [139]. By altering the microtubule dynamics, a prolonged mitosis will take place leading to the activation of the apoptotic pathway [140]. On the other hand, cancer cells with an active SAC can present mitotic slippage, by reaching low levels of Cyclin B1 degradation impeding the Cdk1/CyclinB complex [141]. Cells presenting mitotic slippage will either become tetraploid and stop dividing after G_1 and become senescent or die. A small

percentage of often p53 mutated cancer cells can continue dividing becoming aneuploid due to aberrant mitosis and present genomic instability, therefore being more prone to malignancy with increased drug resistance [85].

Due to the toxicity of anti-microtubule drugs and acquired resistance, other types of compounds and targets blocking the mitotic phase are being developed and researched.

Mps1

Mps1 or monopolar spindle 1, is a kinase involved in the correct biorientation of sister chromatids in the mitotic spindle and regulator of the SAC, that in early mitosis, repairs the miss-attachments between kinetochores and microtubules [137, 142]. Overexpression of Mps1 has been observed in several cancers including breast, pancreatic or liver cancer [143-145]. This overexpression has been correlated to aneuploidy/chromosomal instability [146] but the role in neuroblastoma, the most common paediatric cancer (younger than 1 year old), has not been investigated. Reversine inhibits both Mps1 and Aurora B, with 2-fold higher affinity for Mps1 [147]. Later on, Mps-BAY2a was designed, a synthetic compound presenting higher specificity to Mps1 than Reversine [148].

Cyclophilins

The peptide bonds are usually in trans conformation due to its lower steric energy as the side chains are found 180 degrees from each other and the ribosome is believed to synthesize the peptides in trans form [149]. Due to the special structure of the amino acid proline containing a cyclized side chain, the peptide bond on the NH-terminal side of proline residues can also be found in cis conformation, where both side chains are in the same side of the plane [150]. The isomerization of these peptide bonds is a slow process and requires a high activation energy. These changes of the isomeric state at the prolyl residue can modify the overall protein conformation [151].

Cyclophilins (Cyps) are one of the three families of peptidyl-prolyl isomerase (PPIase) proteins together with parvulins and FK506 binding proteins (FKBPs)[151]. Cyps have the capacity to catalyse the cis/trans isomerization of peptide bonds at the proline residues (Figure 5) [152, 153], facilitating the stabilization of the cis/trans isomerization and decreasing the time necessary for the process. This is important for protein folding, assembly of multidomain proteins and protein signalling processes [88, 149].

Cyps have been conserved throughout evolution and are present in all cell types and organisms [149, 154]. While all cyclophilins share a common domain known as cyclophilin-like domain or CLD of approximately 109 amino acids, they also present differences surrounding the domain linked to specific functions or subcellular compartmentalization [155-157]. All the cyclophilins contain a common fold architecture of eight antiparallel β-sheets, two α-helices and a short α-helical turn containing the active site residue Trp121 in the β6-β7 loop region [154].

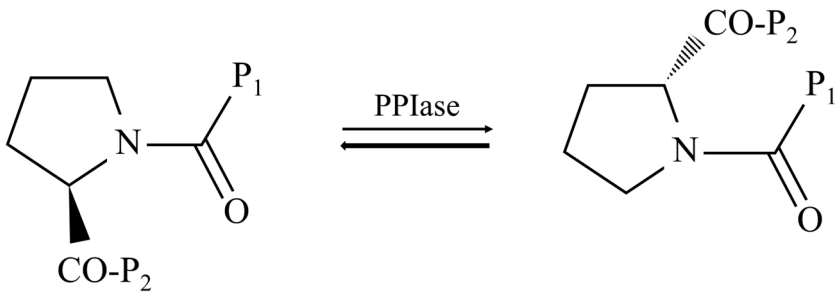

Figure 5. A schematic illustration of cis/trans isomerization of the peptide bond between proline and another amino acid (P_1) and P2 indicates a third amino acid. The isomerization is catalysed by peptidyl-prolyl isomerases like Cyps.

Structurally, two pockets are found in the CLD giving substrate specificity, the first pocket is the hydrophobic catalytic groove where proline is inserted and a conserved arginine at 55 can be found, a highly conserved amino acid needed for the isomerization [151, 158] in addition to a group of hydrophobic, aromatic and polar residues [154, 158-160]. In the CLD domain near the active site, a second deep pocket next to the catalytic groove can be found which surface gives substrate specificity known as the "gatekeeper" and is more unique for each individual cyclophilin [154, 161].

Multiple cyclophilins have been identified in the human genome with different functions and intracellular locations [149, 154]. Cyclophilins A, B and D, the most studied cyclophilins, are involved in several functions including protein folding, protein trafficking and pre-mRNA processing among others [162, 163]. In addition, Cyps can act as molecular switches by inducing conformational changes in the targeted protein affecting their activation or inactivation [149].

Specifically, cyclophilin A (CypA or PPIA), a 18kDa protein, was the first cyclophilin discovered [164, 165]. CypA is the most abundant cyclophilin and is mainly found in the cytoplasm comprising 0.1% of the cytosolic proteins [149, 157]. CypA is involved in protein folding, trafficking, immunomodulation, calcium homeostasis and cell signalling [149, 166, 167]. CypA has also been shown to be important for viral infections [157, 168, 169]. Although CypA is mainly found in

the cytoplasm, it can be secreted by different types of cells in response to inflammatory stimuli, oxidative stress, and cell death, acting as a strong pro-inflammatory signal with chemotactic activity in neutrophils and monocytes through CD147 [167, 170].

Cyclophilin B (CypB or PPIB), 22 kDa, is the most widely distributed protein [171] but is mainly found in the Endoplasmic Reticulum (ER) due to its involvement in protein folding and ER stress response [172]. CypB is also involved in cell signalling [173, 174].

Both CypA and B can be secreted as inflammatory response, although CypB is not able to induce a proinflammatory response alone [157].

Cyclophilin D (CypD or PPIF), located in the mitochondrial matrix, is a key regulator of the mitochondrial permeability transition pore (MPTP) [133]. The MPTP non-specifically transports aqueous solutes in the mitochondria inner membrane (up to 1500 Da), in addition protons can pass freely through the inner membrane also regulating the mitochondrial membrane potential. The MPTP can act as calcium efflux from mitochondria to cytoplasm when opened briefly [175]. The reduced form of CypD is involved in the folding of proteins that are imported to the mitochondria helping to maintain the integrity of the mitochondria. Several stimuli such as increased ROS levels or ATP and ADP depletion can lead to persistent opening of MPTP causing a decrease in mitochondrial membrane potential, inhibition of ATP production and release of cytochrome C to the cytoplasm inducing both necrotic and apoptotic death [115, 176-178]. Specifically, the oxidized form of CypD can open the MPTP [179] by binding to ANT that will change from nucleotide transporter to a non-specific transition pore leading to the persistent opening of MPTP. During this process, it is believed that VDAC molecules will oligomerize and form larger channels leading to release of cytochrome C and other pro-apoptotic factors leading to apoptosis [133].

Classical inhibitors and derivatives

Cyclosporin A (CsA) (Figure 6), the most well-known cyclophilin inhibitor, is a non-ribosomal cyclic undecapeptide. CsA was the first natural cyclophilin inhibitor discovered and first isolated from the fungus *Tolypocladium inflatum* by Sandoz (former Novartis) [180]. Although CsA's intracellular main target is CypA [165], CsA is also able to bind to most of the cyclophilins discovered [154] such as CypB situated in the ER, inducing its secretion outside the cell [181]. CsA is able to inhibit the PPIase activity of the cyclophilins by directly blocking the catalytic side [154]. Currently, CsA is used in the clinic as an immunosuppressant treatment. This effect is due to the ternary formation of cyclophilin A and calcineurin, a calcium-dependent phosphatase regulating the expression of several cytokines in active T cells [28]. This ternary complex inhibits the translocation of nuclear factor of

activated T cells (NFAT) leading to a decreased activity and proliferation of T-lymphocytes [166, 182].

Several cyclophilin inhibitors have been designed based on the structure of CsA via a semisynthetic approach including NIM811 developed by Sandoz [183]. NIM811 is a cyclosporin-based inhibitor not able to inhibit calcineurin, therefore not presenting immunosuppressive activity [184, 185] but still being able to bind to other cyclophilins such as B [186]. NIM811 was shown to inhibit HCV [187].

Alisporivir is a non-immunosuppressive, cyclophilin inhibitor semi-synthetically produced CsA-based not able to bind to calcineurin. As NIM811, Alisporivir inhibits viral replication such as HCV replication [188]. Alisporivir reached Phase II clinical trials as an antiviral treatment against HCV [189].

Sanglifehrin A (SfA) (Figure 6) is a novel natural cyclophilin inhibitor consisting of 22 membered macrocycle isolated from *Streptomyces* bacteria [157, 190] that binds to cyclophilin but is structurally distinct from CsA. SfA has 20-fold higher affinity than CsA to Cyps. Similar to CsA, SfA binds to the active site and is able to inhibit the PPIase activity [190, 191]. SfA presents immunosuppressive activity but is unrelated to calcineurin and CypA like CsA [192].

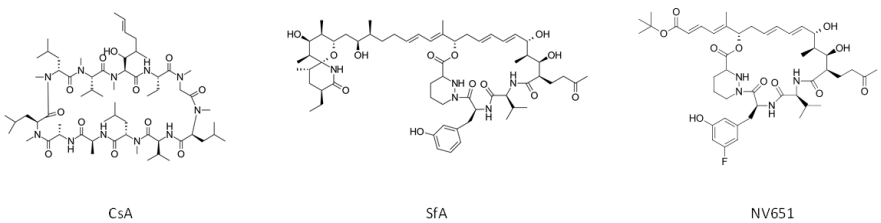

Figure 6. Chemical structures of CsA, SfA and NV651.

NV556, was designed by bioengineering and semisynthetic approach based on the SfA-scaffold. This molecule was designed at Isomerase Therapeutics (formerly Biotica) in collaboration with Abliva (formerly Neurovive Pharmaceuticals). NV556 presented a more potent inhibitory capacity for PPIase activity than CsA. This molecule was also shown to inhibit HBV and HCV *in vitro* and presented a reduced off-target inhibition of previously observed targets in CsA such as drug transporters involved in bilirubin or xenobiotic transporters [193]. When given orally, NV556 accumulated in the liver making this organ an interesting target for the compound [193].

NV651 (Figure 6) belongs to the family of optimized sanglifehrin-based cyclophilin inhibitors together with NV556.

Unfortunately, apart from CsA, none of the cyclophilin inhibitors have advanced to the market yet.

The role of Cyclophilins in liver fibrosis

The potential strategies to treat liver fibrosis have targeted liver inflammation, hepatic stellate cells activation and ECM modification.

Primary biliary cirrhosis (PBC) can reoccur after liver transplantation. Interestingly, patients that were treated with CsA presented a lower risk of developing liver fibrosis while tacrolimus, a more potent immunosuppressant and calcineurin inhibitor that does not interact with cyclophilins [194], resulted in higher risk of developing PBC [195]. This effect seemed to be an indication of the potential effect of cyclophilin inhibition in fibrosis.

Indeed, overexpression of cyclophilins has been observed in fibrotic tissue [170, 171, 196] including human oral submucous fibrosis [196], liver fibrosis [171], troponin I-induced myocarditis [167] and myocardial interstitial fibrosis in post-myocardial infarction (MI) heart failure [197]. Overexpressed cyclophilins such as A and B are involved in several stages of the fibrotic development including inflammation, hepatocyte death and HSC activation [19, 28, 167, 171, 178].

ALT (alanine aminotransferase) and AST (Aspartate aminotransferase) are transaminase enzymes expressed in hepatocytes found in high concentrations in the liver in comparison to other organ tissues [198]. ALT and AST levels can increase in serum due to liver dysfunction and increased cellular necrosis [198], although AST can be produced from other tissues as well. An important point to have in consideration is the need for hepatocyte death to observe elevated levels of AST and ALT [1].

During their studies Wang et al. [171] presented a reduction in serum AST and ALT levels and increased albumin levels upon NIM811 treatment in their CCl_4-model, indicating a reduction in liver necrosis and improved liver function. In addition, Rehman et al. [115] showed that chemical inhibition with NIM811 in the bile duct ligation (BDL) model ameliorated ALT levels, hepatic necrosis and apoptosis in hepatocytes. Acute effect of BDL resulted in depolarization of the mitochondria due to MPTP opening and Cytochrome C release and was ameliorated with NIM811 treatment. Interestingly, CsA treatment in hepatocytes exposed to hydrophobic bile acids had a reduced percentage of apoptotic cells in comparison to the control [199]. Biliary atresia (BA) is a liver disease characterized by obstruction of the bile duct, inflammation, fibrosis and cirrhosis leading to liver dysfunction affecting children up to 3 months old but the cause of the disease has not been characterized [170]. MM284 treatment, a CsA-based non-permeable cyclophilin inhibitor, restored the decreased weight observed in BA mice and decreased bilirubinuria, detection of bilirubin in the urine caused by hepatocellular dysfunction [170].

Acetaminophen can induce hepatocyte death due to glutathione depletion, oxidative stress, and mitochondrial dysfunction. NIM811 and CsA were able to delay acetaminophen-induced necrosis and apoptosis in hepatocytes preventing

mitochondrial depolarization, permeabilization of the inner mitochondrial membrane, mitochondrial swelling, outer membrane rupture and cytochrome C release that would have led to both necrosis due to ATP depletion and apoptosis [178].

The effect of cyclophilin inhibition has been observed in other organs such as the cytoprotective role of CypD ablation in cardiomyocytes from apoptosis-related myocardial infarction [197].

Extracellular CypA, B and C can be secreted to the extracellular space in response to inflammatory stimuli and interact with Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) or CD147 [170, 200], a transmembrane glycoprotein expressed in epithelial, endothelial and hematopoietic cells [201]. This interaction can activate several inflammatory activities such as leucocyte chemotaxis or production of matrix metalloproteinases [202]. Previous studies have shown that extracellular CypA can activate the ERK pathway through interaction with CD147 leading to increase expression of MMPs and CD147 as positive feedback, leading to increased inflammatory response [203, 204]. Studies with MM284 demonstrated to effectively inhibit extracellular CypA in troponin I-induced autoimmune myocarditis, a model developing severe inflammation and fibrosis due to immunization with murine cardiac troponin I. A decrease in myocardial injury was observed upon MM284 treatment. A more detailed examination showed a reduction in inflammation with a lower number of infiltrated T cells and macrophages upon MM284 treatment. When evaluated *in vitro*, monocytes treated with CypA increased their migration and adhesion and this effect was attenuated with the MM284 treatment [167]. In the experimental BA model treatment with MM284, Iordanskaia et al. [170] reported a decrease in the number of infiltrated neutrophils in the liver and decreased inflammatory foci, likely due to the decreased chemotaxis [205]. In their BA model, they also observed an upregulation of TIMP-4 and MMP-7 in the liver and this expression was ameliorated upon treatment with MM284. IL-6 expression, an interleukin secreted by macrophages in response to extracellular cyclophilins [170, 206], was decreased with MM284 treatment, probably due to decreased migration of neutrophils and other inflammatory cells in the liver [170].

In the CCl_4 rat model [171], NIM811 treatment reduced the levels of overexpressed CypB together with CypD leading to a decrease in liver fibrosis. In cardiac fibrosis, extracellular CypA activated CD147 increasing the expression of MMPs such as MMP-9 (a pro-fibrotic MMP [207]) and this effect was ameliorated with MM284 treatment [167].

In addition, correct functionality of CypB was shown to be a necessary factor for the correct folding of collagen type I as mutations of PPIB were correlated to the appearance of Osteogenesis Imperfecta in humans [208]. Confirming the importance of CypB, KO of PPIB resulted in a decreased rate of collagen type I and

III causing an overmodification of lysyl residues as well as increased intracellular degradation of these two types [209, 210].

CypD inhibition with CsA and SfA decreased fibrosis by decreasing proliferation in cardiac fibroblasts [197]. This effect seemed to indicate a potential role in the fibroblast's activation and potential involvement in HSC activation. Indeed, *in vitro,* NIM811 and CsA presented an anti-fibrogenic effect by decreasing HSC proliferation without induction of cell death, decreasing collagen production, and increasing collagenase activity. A detailed examination showed that NIM811 was able to inhibit Smad2/3 phosphorylation with increased Smad7, a negative regulator of the TGF-β pathway, and decreasing TGFBRI transcription [28]. NIM811 treatment in the CCl_4-model decreased the expression of collagen type III, TIMP-1 and TGF-β, factors important for the modification of the ECM. In addition, staining of α-SMA in HSC was decreased with NIM811 treatment in comparison to control [171].

Proliferation and collagen production can be regulated by MAPK signal cascades [211], this cascade includes factors such as ERK1/2, JNK and p38. In HSC, extracellular CypA increased the phosphorylation of Smad2/3 and this post-translational modification was inhibited with MM284 treatment. A cross-talk between TGF-β, CD147, SMADs and ERK was believed to happen in the liver [170]. CsA was able to decrease cell proliferation and collagen production in HSC inhibiting JNK activation and ERK1/2. In addition, a decrease in TIMP-1 concentration and increase in MMP-1 activity was shown upon CsA treatment resulting in increased collagenase activity [212].

A recent publication, used CRV431, a CsA non-immunosuppressive analog able to inhibit the PPIase activity of CypA, B, D and G more potently than CsA. Treatment with CRV431 presented a decrease in liver fibrosis in the CCl_4-model and in the NASH mouse model induced by a single injection of streptozotocin followed by high fat diet. CRV431 presented decreased liver fibrosis in both models while obeticholic acid, used as reference drug, had no effect. Interestingly, at week 30 several tumours could be detected in this NASH model treated with vehicle with nodules bigger than 1 cm while CRV341 treatment presented a reduced number of tumours and smaller in size [213]. These data added an interesting link between cyclophilins and NASH and HCC development.

The role of Cyclophilins in cancer

Cyclophilin overexpression such as A and B has been reported in several types of cancer including HCC, where its overexpression has been linked to several activities including cancer proliferation, inhibition of hypoxia-induced apoptosis, metastasis, resistance to chemotherapy, and cell cycle progression [75, 91, 157, 214-217].

Interestingly, studies have shown a positive correlation between cyclophilin expression and HCC [75].

Cyclophilins can promote proliferation in several types of cancers including cholangiocarcinoma, gastric, breast cancer or HCC [88, 218-222] while chemical inhibition with SfA and CsA results in anti-proliferative effect in HCC cell lines with no increased cell death indicating a cytostatic effect rather than cytotoxic in this case [217]. On the other hand, silencing of CypA was shown to decrease cancer cell proliferation and induce apoptosis in NSCLC *in vivo* [223]. Animal models have shown that inhibition of cyclophilins reduced tumor growth in gastric cancer [216], NSCLC [224], cholangiocarcinoma [219] and HCC [214].

Several publications have investigated the potential regulatory mechanisms behind cyclophilin activity during cancer progression.

CypA can stimulate cell proliferation through binding of CD147 leading to activation of ERK1/2 [225, 226]. In HCC, CypA was found to be a target of Ubiquitin-specific protease 4 (USP4), a deubiquitinating enzyme important for cancer. The interaction between USP4 and CypA inhibited CypA degradation and led to activation of MAPK signalling with increased phosphorylation of ERK1/2, increasing cell proliferation in HCC *in vitro* [221].

Cyclophilins are also involved in the cell cycle progression facilitating the G_1/S transition [75, 218, 222]. Interestingly, in HCC [215], gastric cancer [216] and cholangiocarcinoma [219], CypA increased proliferation, through CD147 activation, inducing the phosphorylation of ERK1/2 and promoting G_1/S transition. This effect was caused by the increase of cyclin D1 levels and phosphorylation of retinoblastoma (RB) facilitating the G_1/S transition [216, 219] while no changes in cell viability were observed [215, 216].

In HCC, cyclophilin J or PPIL3 (Peptidyl-Prolyl Isomerase-Like 3) inhibition caused an increase in G_1 and reduction in S population. CypJ promoted G_1/S transition by direct binding to Cyclin D1 and promoting its upregulation. A downregulation in p27 expression was also observed with overexpression of PPIJ [214].

In NSCLC, cell viability and cell proliferation were decreased with chemical inhibition of CypA by HL001 treatment. This inhibition caused disturbances in the cell cycle with a block of cells in the S phase. This effect was correlated with a reduction in cell cycle proteins such as Cyclin D1, Cyclin E, CDK2 and Cdc25A. This block was followed by activation of the apoptotic pathway with increased pro-apoptotic factors such as Puma, Noxa and Bax. HL001 treatment increased p53 and p21 protein levels, reducing the degradation of p53 by blocking the binding of MDM2 to p53 inhibiting its ubiquitination. HL001 effectivity was dependent on the polymorphism at codon 72 with Arginine [224]. Interestingly, Baum et al. [130] presented the interaction between Cyclophilin18 (CypA) and p53 through CypA's

active site. This interaction led to blockage of $p53^{P72}$ from inducing transcription of genes such as GADD45a. CsA treatment or Cyp18 KO increased apoptosis in combination with daunorubicin, a chemoagent activating p53-dependent apoptosis. Single treatment with CsA presented disturbances in the cell cycle by increasing the percentage of cells in the G_2/M phase and decreasing G_1 but no clear apoptosis was observed [130].

CypA silencing blocked cells in G_2/M phase with increased apoptosis in NSCLC. Irradiation induced an upregulation of CypA and its inhibition led to a higher accumulation of cells in G_2/M as well as increased apoptotic population [227], indicating a potential role in therapy resistance.

Chemical inhibition with CsA or SfA presented a synergistic effect with cisplatin increasing cell death in HCC and ovarian cisplatin-resistant cancer cells. Authors have hypothesized that the inhibition of the antioxidant activity and potential decreased expression of genes involved in DNA damage repair might partially explain this synergy [217, 228].

Indeed, cisplatin treatment in cisplatin-resistant ovarian carcinoma presented increased expression of DNA damage repair enzymes while CsA treatment reduced this effect [229]. In an immortalized human epidermal keratinocyte presenting a mutated p53, CsA blocked UVB-induced DNA damage repair. This was believed to be through inhibition of the expression of xeroderma pigmentosum C (XPC) involved in the GG-NER. CsA induced the activation of the PI3K/Akt pathway leading to translocation of E2F4/p130, a repressor of the XPC transcription, into the nucleus blocking then transcription of XPC [123]. In human ovarian carcinoma *in vitro,* CsA treatment decreased the expression of ERCC1, a key factor in the DNA repair through NER and linked to cisplatin resistance [230]. Interestingly, they report a minimal effect on cell growth upon individual treatment with CsA but significantly decreased proliferation in combination with cisplatin and increased cytotoxicity [87].

ROS is a group of free radical species including superoxide or non-radical species like hydrogen peroxide and is mainly formed in the mitochondria. Interestingly, cancer cells can acquire resistance to some chemoagents such as cisplatin by blocking ROS-induced cell death. In HCC cell lines, upregulation of CypA was observed upon cisplatin treatment [217]. CypA was shown to inhibit oxidative-induced apoptosis [231] while treatment with CsA or SfA or CypA silencing in combination with cisplatin increased cell death correlated to an increase in ROS levels and decreased glutathione in glioma cells [232] and HCC [217].

In NSCLC cells, HL001 treatment led to elevated ROS followed by increased γH2AX levels, potentially indicating DNA damage due to increased ROS. Combination with cisplatin in $p53^{72R}$ cells presented a synergistic antiproliferative effect with HL001 *in vitro*. In NSCLC orthotopic model, HL001 together with cisplatin were able to reduce tumor growth with no changes in body weight [224].

CypB expression increased upon H_2O_2 treatment protecting against oxidative stress and decreasing ROS-induced apoptosis in HCC *in vitro*. This effect was a result of increased secreted CypB activating CD147, phosphorylating ERK and other factors of the ERK pathway such as Ras, Raf and MEK, protecting the cells against ROS-induced apoptosis. Cisplatin also increased the secretion of CypB levels in comparison to non-treated while CsA inhibited the activation of this pathway [233].

ROS levels can be decreased by the activity of antioxidant enzymes. Unbalance between ROS levels and antioxidant activity can lead to oxidative stress with lipid, protein, and DNA damage. CsA is known to increase ROS levels and lipid peroxidation [234, 235]. In HCC cell lines, cyclophilins decreased H_2O_2-mediated ROS levels restoring the activity of enzymes important for the antioxidant activity such as superoxide dismutase (SOD), that transforms superoxide into hydrogen peroxide, catalase (CAT) and glutathione peroxidase (GPx) transforming H_2O_2 to H_2O and O_2, and thioredoxin (TRR), involved in redox balance, protecting against mitochondrial dysfunction [235].

HCC is usually developed in a dysfunctional cirrhotic liver with altered blood supply causing local hypoxia. As a solid tumor, HCC usually contains a hypoxic microenvironment. To avoid cell death, cancer cells can activate the hypoxia inducible factor 1 alpha or HIF-1α that can lead to transformation into malignant, metastatic and chemoresistant cancer cells [91, 217]. Hypoxic conditions induced an upregulation of CypA but treatment with either CsA or SfA resulted in a reduction in cell viability in HCC [217]. CypB expression is upregulated with HIF-1α creating a positive loop by upregulating HIF-1α itself. This positive loop seemed to be related to CypB translocation from the ER to the nucleus and its interaction with STAT3 that led to increase transcription of HIF-1α. CypB was able to protect cancer cells against hypoxic-induced apoptosis and cisplatin treatment in a p53-independent manner. Both Cisplatin and hypoxia can increase ROS levels, and this was ameliorated by CypB. Interestingly, in a HEPG2 and HUH7 xenograft mice model expressing CypB and treated with cisplatin, mice presented an increased tumor growth in comparison to the ones with silenced CypB. CypB expression also increased VEGF, EPO, and GLUT1, hypoxia-induced genes leading to increased angiogenesis and glucose metabolism [91]. In gastric cancer, CypB silencing caused a downregulation in VEGFR expression, also involved in angiogenesis [222]. Corroborating their role in hypoxia resistance, HIF-1α increased the expression of CypA in a human prostate cancer *in vitro* through binding to HRE or hypoxia response elements and by inhibiting hypoxia-induced and cisplatin-induced apoptosis independent of p53 status. Increased ROS levels due to hypoxic conditions, cisplatin or H_2O_2 treatment were attenuated with CypA protecting against mitochondrial membrane potential depolarization and decreasing cytochrome C release [163].

In addition, cyclophilins were found to upregulate other genes involved in chemoresistance such as ABC transporters and glutathione S-transferases, involved

in drug transport and metabolization, decreasing the intracellular concentration of compounds and potentiating therapy resistance [88, 236, 237]. Overexpression of CypA and J was linked to increased resistance of doxorubicin and vincristine in comparison to normal expression with reduced intracellular accumulation of doxorubicin when CypA or J were overexpressed [236].

Cyclophilins are also involved in cell death specifically in the activation of apoptosis in several types of cancers [238]. In colon cancer cells, CypA was shown to inhibit the caspase-dependent apoptosis by sequestering cytochrome C [239].

CypD function in cancer seems to be controversial while some articles present a protective role of CypD against apoptosis in glioma cells [240], other publications show the need of a functional CypD for the activation of cell death through the opening of MPTP when treated with cytotoxic reagents. Emodin treatment in HCC *in vitro* led to apoptosis while CsA inhibited this effect and blocked cytochrome C release [179]. In addition, CypD was shown to interact with p53, leading to opening of the MPTP [241]. *In vitro*, Sorafenib treatment increased CypD expression, increasing apoptosis and decreasing mitochondrial membrane potential in clear cell-renal cell carcinoma [242]. In an *in vitro* study with HCC cell lines, the sensitization of doxorubicin-resistant cell lines with Rhein was inhibited with the use of CsA blocking the MPTP opening [133].

Although CypD is an important key regulator of the MPTP and its inhibition in normal cells has shown a cytoprotective role, several publications have concluded that the overexpression of CypD in cancer cells results in an anti-apoptotic effect. This is believed to be through Bcl-2 binding and inhibiting the release of cytochrome C from the mitochondria, independently of its role in MPTP regulation [243]. Another study showed that CypD was stabilized by oncogenic Ras through Raf-1/MEK/ERK pathway leading to tumor progression and increased colony formation. CypD inhibition with CsA inhibited tumor formation in K-Ras xenografted lung cancer models. Specifically, CypD decreased p21 and p53 activation leading to decreased senescence. They could also detect the interaction in the mitochondria between p53 and CypD while CsA was able to block this interaction decreasing tumor formation [244].

Other studies have presented, the correlation between CypB protein levels to lymphatic node metastasis in gastric cancer [218] and CypA expression in HCC cell lines to increased metastatic activity *in vitro* [245]. In addition, *in vivo* CypA increased metastasis to the lung with cells injected intravenously in the tail [245] indicating a potential role of cyclophilins in metastasis. Metastasis is a multi-step process including adhesion, invasion and migration and ECM degradation. As previously mentioned, degradation of the ECM can be mediated though different molecules such as matrix metalloproteinases. CypA can increase HCC adhesion and migration and invasion capacities by increasing the expression of MMP-3 (also important in EMT) and MMP9. As potential hypothesis, authors described the

involvement of CD147 activation in the expressions of MMP3 and 9 [245]. Interestingly, CD147 has also been linked to EMT and cytoskeleton rearrangement in cancer cells, leading to increase tumor growth and metastasis [66, 246]. In HCC cell lines, CypA led to hypo-phosphorylation of CD147, altering the ECM and increasing cell migration and invasion [66]. Other cyclophilins such as Cyclophilin C mainly residing in the ER was also able to bind to CD147 activating AKT1/2 and MMP-2 increasing migration and invasion in breast cancer cell lines [247].

The immune system is an important factor controlling tumor growth and metastasis. CD147 inhibition in xenograft mouse model led to decreased tumor growth with a higher infiltration of immune cells such as T cells. *In vitro* co-culture with T cells and CD147 silenced cancer cells resulted in a decreased cell viability in HCC cells than in cancer cells expressing CD147. In addition, T cells chemotaxis was increased with extracellular CypA and with co-culture of cancer cells not expressing CD147, probably due to the uptake of CypA from the cancer cells for their own benefit, decreasing therefore the infiltration [215].

Cyclophilins have also been involved in other activities such as splicing and ER stress response. In HCC *in vitro*, CypA interacted with SR-25 (serine/arginine-rich-25), an alternative splicing modulator. Both CypA and SR-25 co-localize in the nucleus and CypA increased the expression of SR-25. It was hypothesized that CypA was needed for the different conformations of SR-25 during splicing cycle and alter their conformation [248].

In addition, increased accumulation of misfolded proteins in the ER induces ER stress leading to increased apoptosis. ER stress activated CypB expression. Block of CypB increased calcium leakage from ER and ROS generation, decreasing mitochondrial membrane potential and inducing cell death. Therefore, CypB could protect against ER stress [172].

The present investigation

Paper I: Evaluation of NV556, a Novel Cyclophilin Inhibitor, as a Potential Antifibrotic Compound for Liver Fibrosis

Aim

The aim of paper I was to investigate the antifibrotic effects of NV556, a novel potent sanglifehrin-based cyclophilin inhibitor, *in vitro* and *in vivo*.

Results and discussion

NASH, the severe form of NAFLD, is characterised by steatosis, hepatocyte death, inflammation and formation of liver fibrosis [249]. Cyclophilins play an important role in the development of chronic liver diseases including chronic inflammation, hepatocyte death and HSC activation [28, 167, 171, 178].

We initially evaluated the potential effect of NV556, a novel sanglifehrin-based potent cyclophilin inhibitor with the former name of NVP018 [193] in two well-established NASH models.

The MCD model was induced with a methionine and choline-deficient diet for eight weeks. After one week of diet, mice were separated and treated orally with vehicle, 100 mg/kg of NV556 or 30 mg/kg of obeticholic acid as a reference drug for seven weeks. A weight loss of 5 grams was seen before the beginning of the treatment as previously observed [114].

Throughout the experiment, no differences in weight were observed between groups. AST and ALT levels were examined three times throughout the treatment period. At 7 weeks of treatment a decrease was observed for both AST and ALT levels in NV556 treated mice, although no significant effect was observed in liver to body weight ratio, liver total cholesterol, triglyceride, or fatty acids. No significant changes were observed in lobular inflammation, ballooning or steatosis but a significant reduction was observed in the Sirius red positive areas indicating a decrease in fibrosis with NV556 treatment.

CypD downregulation, a key component and regulator of the MPTP, has been shown to protect tubular cells from apoptosis in the unilateral ureter obstruction model of renal interstitial fibrosis [250]. In addition, chemical inhibition with NIM811 has also resulted in a cytoprotective effect decreasing necrosis *in vivo* in both bile duct ligation (BDL) and CCl_4 treatment model as well as a delay in acetaminophen-induced necrotic cell death in hepatocytes [115, 171, 178].

Interestingly, Wang et al. [171] reported a significant decrease of liver fibrosis and ALT and AST levels in serum without changes in steatosis, suggesting a potential cytoprotective role in hepatocytes without altering the rest of the NAFLD parameters histologically analysed.

We then proceeded to evaluate the effect of NV556 on the STAM model. As previously mentioned, this model is induced by the combination of streptozotocin and high fat diet (HFD). After one week of HFD, mice were divided and orally treated with vehicle, NV556 or Telmisartan, used as a reference drug. Body weight was not significantly changed in any of the treatment groups and no changes were observed in NV556 treatment group in comparison to vehicle for liver to total weight ratio.

NV556 treatment presented a reduction in whole blood glucose at six weeks but no differences were observed at seven weeks. No clear differences were observed for plasma triglycerides or insulin levels upon NV556 treatment. NAS score was used for histological evaluation of the liver, resulting in no changes in steatosis, hepatocellular ballooning, or inflammation but a significant decrease in Sirius red staining indicating a decrease in fibrosis. Therefore, we could confirm the antifibrotic potential of NV556 in both STAM and MCD models. This antifibrotic effect was also observed in an independent setup for the STAM model [251].

As both the STAM and MCD models presented a reduction in liver fibrosis upon NV556 treatment, we decided to evaluate the potential activity against HSC, since they play a major role in collagen deposition. To do so, we evaluated the effect of NV556 in a new *in vitro* 3D model developed by Mazza, Al-Akkad [252], that mimic the native ECM structures. This was performed by an optimized decellularization technique able to isolate the ECM from healthy human liver tissue while maintaining the properties and structure of the ECM.

We examined the effect of early treatment where TGF-β activation and NV556 were given at the same time and late treatment where NV556 was given two days after TGF-β activation in LX2 cells, a well characterized human HSC line engrafted in 3D liver scaffolds. We evaluated the effect on gene expression in unstimulated LX2 cells, showing a downregulation of LOX and collagen type I alpha I chain (COL1A1) in both early and late treatment. ELISA assay demonstrated a decreased secretion of procollagen I for both early and late-NV556 treatment.

LX2 activated cells by TGF-β presented a reduction in LOX expression in both NV556-early and late treatment in comparison to control and COL1A1 expression for early treatment. ELISA also confirmed a decrease in procollagen type I secretion for both conditions.

NV556 was titrated at a concentration of 0, 0.2, 1 or 5 μM in TGF-β stimulated or unstimulated showing a dose-response effect in LX2 cells after 24 hours of treatment with a decrease in LOX, COL1A1 and COL4A1(collagen IV) but no changes were observed in markers of HSC activation, indicating that NV556 might directly decrease the expression of COL1A1 and LOX.

Chemical inhibition with NIM811 or downregulation of cyclophilins such as CypB and CypD can decrease collagen production such as collagen type I or III [28, 171], presenting similarities to our 3D *in vitro* model where a downregulation of collagen type I was also observed. On the other hand, NV556 treatment resulted in a downregulation of collagen type IV while collagen type III was not affected. CypB is involved in the correct folding of collagen type I where its inhibition or deficient functionality can lead to over-modification of lysyl residues increasing its degradation [208-210]. Although we did not evaluate the potential effect of cyclophilin inhibition in collagen folding, this effect could be an explanation for the decreased secretion of procollagen type I with NV556 treatment.

LOX, a marker of activated HSC, is a key factor for the cross-linking of collagens and elastin increasing therefore the stiffness of ECM [46, 253, 254]. LOX inhibition was shown to reduce the accumulation of cross-linked collagens [253]. The downregulation observed in our *in vitro* 3D model could therefore contribute to the antifibrotic effect observed in the STAM and MCD models upon NV556 treatment.

Conclusions Paper I

The data published in paper I presents NV556 as a potential candidate for the treatment of liver fibrosis due to its effect observed in both models tested, STAM and MCD model. We could also confirm in the *in vitro* 3D model the decreased production of collagen and LOX, potentially interfering in the crosslinking of collagens and elastin during the fibrotic development.

Paper II: Novel Cyclophilin Inhibitor Decreases Cell Proliferation and Tumour Growth in Models of Hepatocellular Carcinoma

Aim

We explored the potential of cyclophilin inhibitors as new therapeutic options for HCC.

Results and discussion

Cyclophilins are overexpressed in several types of cancer [157, 216, 218] including HCC [75, 91, 214], playing an important role in several cancer activities such as tumour growth [75, 215, 216], metastasis [255], resistance to radiation and chemotherapy [91, 256]. We could also confirm the upregulation of cyclophilins such as PPIA and PPIB in comparison to normal tissue and correlation between the expression of these cyclophilins with prognosis in HCC patients with the use of Kaplan-Meier curves retrieved from GEPIA [257].

We then screened several cyclophilin inhibitors based on the sanglifehrin scaffold. This platform includes NV556, previously described in Project I for the antifibrotic activity in liver fibrosis. To select the best candidate to perform further studies, we evaluated the dose-response effect of several cyclophilin inhibitors in HUH7 cells using Acumen proliferation assay. This presented NV651 as the most interesting candidate due to its effect in proliferation.

NV651 potency to decrease peptidyl-prolyl isomerase activity was evaluated in a cell-free assay, demonstrating a higher capacity to inhibit the PPIase activity of CypA, CypB, CypD and Cyp40 than CsA or SfA. Previous publications have demonstrated the capacity of CsA to decrease proliferation in HCC at the μM range [214]. On the other hand, CsA presents immunosuppressive activity [166] presenting potential difficulties to be used in the clinic as a treatment against cancer. Due to the higher PPIase activity of NV651 in comparison to CsA and the lack of this immunosuppressive activity [258], we believed NV651 was a better candidate for HCC.

The next step in our study was to evaluate whether NV651 could be more potent than sorafenib, one of the few treatments approved at advanced HCC [98]. To do so, we treated 9 HCC cell lines against sorafenib or NV651 resulting in a higher anti-proliferative activity of NV651 in 8 out 9 HCC cell lines. Clonogenic assays indicated a reduction of the colony numbers and detailed proliferation assays using HEPG2 and HUH7 confirmed NV651 anti-proliferative activity at the nM range.

One of the key studies for the evaluation of a compound as a potential treatment is the confirmation of its safety in normal cells. Therefore, we used a panel of assays with CellTiter Glo to evaluate the potential *in vitro* toxicity in monocytes–macrophages, dendritic cells, bone marrow progenitor cells, hepatocytes, human-induced pluripotent stem cell (iPS) cardiomyocytes, iPS neurons and primary renal proximal tubule epithelial cells (RPTEC). Some toxicity could be observed at the μM range with IC50 values of 24.3, 24.2 and 20.9 μM against monocytes, dendritic cells, and kidney cells, respectively.

Detailed examination of potential toxicity in primary hepatocytes presented that the lowest minimum effective concentration (MEC) caused a decrease in the glutathione (GSH) content and an increase in ROS levels as the lowest (AC50, concentration in which a 50% maximum effect is observed for each cell health parameter) both at the μM range. Having in consideration that the antiproliferative effects were observed at nM range, we could confirm a wide safety margin for NV651 treatment.

No effect on body weight resulted from NV651 via intraperitoneal (IP) after 5 days of treatment at 25 mg/kg, 50 mg/kg or 200 mg/kg. NV651 could also be administrated either intravenously or orally as these two administration routes presented blood levels in mice with peak concentrations of 5 and 1 hour respectively. NV651 was still detected in liver after 24 hours of treatment with a threefold-higher concentration in liver than in blood with 2% oral bioavailability. These data show that NV651 is an orally bioavailable compound that accumulate in the liver, presenting the liver as an optimal target.

As NV651 presented a potent antiproliferative effect, we wanted to know whether this could be caused by cytotoxic activity, to evaluate this we performed several assays in HEPG2 and HUH7 cells. A long-term exposure with evaluation of membrane integrity by DAPI staining, a non-permeable dye, showed significant differences in cell viability up to 10 days in HUH7 cells. At a shorter treatment exposure, 72 hours, no effect was observed in membrane integrity with PI staining or mitochondrial membrane potential, an early apoptotic marker, analysed by flow cytometry in HEPG2 and HUH7 cells. The DNA cleaved population or $SubG_1$ was also evaluated up to 96 hours, here we could observe a statistically significant increase in the $subG_1$ population in NV651 treated HEPG2 and HUH7 cells. These results suggest that NV651 might have a small cytotoxic effect after a long-term exposure, but the potent anti-proliferative effect could not be totally explained by this mild cytotoxic effect.

Due to the potent antiproliferative effect and the low effect on cell death we wanted to know whether this effect could be related to a disturbance in the cell cycle. When evaluating the potential cell cycle effect up to 96 hours of treatment in HEPG2 and HUH7 cells by PI staining and flow cytometry, we observed a clear G_2/M block in both cell lines. To know at what phase the cells could have been accumulated, we

evaluated the percentage of cells presenting a phosphorylation in the histone 3 (PH3), a marker of mitosis. The population positive for PH3 was increased in both cell lines with NV651 treatment. In addition, we quantified the number of cells in the mitotic phase using tubulin and DAPI staining and confocal microscopy after 48 hours in HUH7 cells confirming the mitotic block upon NV651 treatment. Cyclophilins have been previously shown to be involved in the cell cycle progression in gastric cancer and cholangiocarcinoma as well as HCC, being necessary for the progression from G_1 to S phase [75, 214-216, 218]. On the other hand, CypA knockdown has also induced a G_2/M arrest in lung adenocarcinoma cells [227].

We evaluated the *in vivo* efficacy in 20 female athymic nude mice xenografted with $1x10^6$ transduced Luc^+ HEPG2 cells in the right flank. After one week, we already observed a good luminescence signal (1.5×10^8 photons/s) using the *in vivo* imaging system, IVIS. Mice were then separated in control or NV651 treatment groups and treated daily via subcutaneous injection (SC) with 10 mg/kg of NV651 or equal volume of the vehicle up to 3 weeks. Weight was assessed twice a week with no significant differences between vehicle and treatment, confirming the safety of NV651 treatment *in vivo*. Caliper measurements were performed twice a week once the tumour was visible, presenting a decrease in the treatment group in comparison to the vehicle. In addition, IVIS was performed every week since the start of the treatment confirming the reduction in tumour growth observed with the caliper measurements.

Paper III: Synergistic effects of sanglifehrin-based cyclophilin inhibitor, NV651, with cisplatin in hepatocellular carcinoma

Aim

Project III is a follow up on project II with the aim of studying the mechanism of action of NV651 in HCC and the potential synergistic effect of NV651 in combination with other approved HCC treatments.

Results and discussion

We have previously studied the effect of NV651 in HCC cell lines and the block in the mitotic phase. In this study, we evaluated the anti-proliferative effect of NV651 in a set of 50 cancer cell lines including 10 colorectal cancer, 31 liver cancer, and 9 pancreatic cancers. This resulted in broad anti-proliferative effect of NV651 against

different cancer cell lines. We then differentiated cancer cell lines depending on its sensitivity separating sensitive and resistant cells and compared the available transcriptomes either collected from the TCGA database or sequenced with Illumina platform. Gene set enrichment analysis of the available transcriptomes presented several DNA damage repair pathways enriched when comparing sensitive against resistant cancer cell lines such as transcription-coupled or global-genome nucleotide excision repair (TC-NER and GG-NER). In addition, 18 genes were selected as signature genes with an accuracy for the prediction of sensitivity of 89.6%.

We then investigated the early changes of NV651 in HCC after 4 hours of treatment by analysing changes on gene expression in HEPG2 cell lines in comparison to the control group. We also compared the gene expression changes to the CsA treated group to understand the differences observed previously between CsA and NV651 treatment.

Evaluation of Reactome and Gene Ontology of biological processes presented downregulation on gene sets involved in the cell cycle including mitotic phase and DNA replication and DNA damage repair such as homologous recombination or interstrand crosslink repair. Previous publications have shown the downregulation of DNA damage repair upon CsA treatment [123, 228] confirming our results. On the other hand, when comparing changes between CsA and NV651 treated group we could also observe a further downregulation in gene sets involved in DNA damage repair. Gene sets related to the mitotic phase were also downregulated when comparing these two groups, being a potential explanation to the exclusivity of the mitotic block of NV651 in HCC cell lines that have not been previously reported.

Quantitative PCR confirmed the downregulation of genes involved in DNA damage repair specifically in interstrand crosslinks such as BRCA1 or the Fanconi Anemia family in both HEPG2 and HUH7 cells after 4 hours of treatment.

Cyclophilins are involved in therapy resistance through different mechanisms such as increased expression of ABC transporters, that would facilitate the decrease of drug accumulation in the cell [236]. We then proceeded to evaluate the potential synergistic effect of NV651 and already approved HCC treatments. The evaluation of the potential synergistic effect with sorafenib, one of the few available options for advanced HCC resulted in a clear synergistic effect with NV651 in HEPG2 cells after 7 days of exposure but no clear decrease in cell viability was observed by quantification of the membrane integrity and decrease in mitochondrial membrane potential after 72 hours of combination treatment.

Cisplatin, a chemoagent that can be given at an intermediate stage at HCC, produces different types of DNA damage including inter and intrastrand crosslinks in mitochondrial and nuclear DNA as well as increase of ROS levels. The cell survival will depend on the capacity of the cell to repair this DNA damage in relation to the amount of DNA damage level, an ineffective DNA damage repair will therefore lead to the activation of the apoptotic pathway [259, 260].

The main cytotoxic effect of cisplatin is believed to be due to stalling forks in the S phase therefore repair at this phase through Fanconi Anemia pathway and homologous recombination, with key factors such as BRCA1/2, is a key activity for their survival [261, 262]. The importance of the proper functionality of factors involved in these pathways have been confirmed when defective or downregulated Fanconi Anemia or homologous recombination including BRCA1/2 in cancers such as ovarian and pancreatic have presented a better outcome with platinum-based drug therapies increasing its sensitivity [263-266]. Therefore, with our results indicating a downregulation of genes involved in Fanconi Anemia or homologous recombination with NV651, we believe this compound could be an interesting candidate for the combination with chemoagents such as cisplatin. We then decided to test several chemoagents, resulting in cisplatin as the most interesting candidate due to its synergistic effect in cell viability in both HEPG2 and HUH7 cell lines after 72 hours of treatment. We continued with the evaluation of the intrinsic apoptotic pathway by evaluating mitochondrial membrane potential, cleaved DNA or $SubG_1$ and membrane integrity, showing a significant increase with the combination of NV651 and cisplatin in comparison to the individual treatments for both HEPG2 and HUH7 cells.

Due to the previous results presenting a synergistic effect on viability and the downregulation of DNA damage repair mechanisms such as the Fanconi Anemia pathway and homologous recombination, we wanted to know whether the combination of cisplatin and NV651 could influence the capacity of the cells to repair interstrand crosslinks. To evaluate this, we pre-treated HEPG2 and HUH7 cells with NV651 for four hours followed by 2 hours of treatment with cisplatin, we then replenished the cells with fresh media to evaluate their DNA repair capacity by performing an alkaline comet assay. This resulted in a decrease in the repair of interstrand crosslinks when cells were pre-treated with NV651 in comparison to single treatment of cisplatin.

As previously mentioned, the inefficacy of the cells to properly repair interstrand crosslinks could lead to disturbances in the cell cycle caused by fork stalling during DNA replication in the S phase. Therefore, we proceeded to evaluate potential effects on the cell cycle with this combination treatment. Surprisingly a clear G_1 block in both HEPG2 and HUH7 cells was observed upon the combination treatment. This could be explained by the different pathways that the cell would activate to repair interstrand crosslinks depending on the cell cycle phase. When cells are in G_1, two repair pathways can be activated, the transcription-coupled nucleotide excision repair (TC-NER) and the global genome nucleotide excision repair (GG-NER)[267]. TC-NER, is important for the cisplatin resistance [267] and a defective TC-NER could increase the sensitivity to cisplatin [89, 267-269]. TC-NER is involved in the repair of regions that need to be transcribed from the genome [270]. TC-NER is independent of Fanconi Anemia and homologous recombination, although defects at both mechanisms present an additive effect in cell viability as

TC-NER can reduce the burden of interstrand crosslink repair at the S phase [269]. It was previously shown that cancer cells treated with cisplatin and presenting a defective NER are accumulated in the G_1 phase, and this caused an increase in the subG_1 phase [271], presenting similar results with the combination treatment of NV651 together with cisplatin.

Conclusions of Paper II and III

To conclude, we describe here the development of NV651, a novel cyclophilin inhibitor for liver cancer treatment. NV651 showed potency at inhibiting proliferation of HCC cell lines via cell cycle perturbations and arrest of the cells in the mitotic phase. In addition, NV651 was able to decrease tumour growth in a xenograft HCC mouse model. We could also confirm the safety in normal cells and good oral bioavailability. NV651 was able to affect several pathways in HCC in vitro including cell cycle and DNA repair. Combination of NV651 and cisplatin resulted in a potential synergistic effect on cell viability with a significant increase on cell death in comparison to individual treatment. We could also observe a disturbance in the cell cycle and a decrease in the capacity of the cell to repair interstrand crosslinks in comparison to individual cisplatin treatment. Taken these data together, we propose NV651 as a potential treatment against HCC individually or in combination with cisplatin, as lowering the dosage for chemotherapy agents could minimise the risk of acquiring drug resistance and side effects.

Paper IV: Inhibition of mitotic kinase Mps1 promotes cell death in neuroblastoma

Aim

The aim of project IV was to evaluate the effect of Mps1 inhibition in Neuroblastoma and its potential as a molecular target for treatment.

Results and discussion

Neuroblastoma is the most common paediatric cancer in children younger than one year [272] accounting to 10 % of cancer related deaths in population younger than 20 years [273]. The treatment of patients in high-risk is still problematic with limited treatment options and potential toxicity of the therapies [274]. A new interesting approach for the treatment of neuroblastoma has been the targeting of key factors in

the mitotic phase [136], as mitotic factors are overexpressed in several paediatric cancers such as high-risk neuroblastomas [275-277].

We, therefore, wanted to evaluate the potential inhibitory effect of Mps1, a key regulator of the spindle assembly checkpoint, as a potential treatment in neuroblastoma.

As previously mentioned, overexpression of Mps1 has been observed in several cancers including breast, pancreatic or liver cancer [143-145]. This overexpression was correlated to aneuploidy/chromosomal instability [146], but no studies on Mps1 in neuroblastoma have been previously performed.

Overexpression of Mps1 was linked to advanced stages in neuroblastoma. Tumour risk, progression, mortality and MYCN amplification, a poor prognostic marker, were also correlated to Mps1 expression. In addition, Kaplan-Meier curves indicated correlation between Mps1 overexpression and poor prognosis, decreased free survival and overall survival probability. This indicates that Mps1 expression could be used as a prognostic marker for neuroblastoma.

We then evaluated the effect of Mps1 inhibition in cell death specifically activation of the intrinsic apoptotic pathway. To do so, we used different neuroblastoma cell lines including neuroblastic or more malignant differing in MYCN amplification, and ALK and TP53 mutation status. Cell lines were treated for 72 hours with either DMSO as control or chemically inhibited for Mps1 with Mps-BAY2a or Reversine. We initially evaluated cell membrane integrity by PI staining and mitochondrial membrane potential with DiOC6(3). All cell lines presented decreased cell viability upon Mps1 inhibition. Due to the MYCN amplification, TP53 mutated and sensitivity upon Mps1 inhibition, we decided to evaluate the effect on SK-N-Be2c cell line. Due to potential unspecific bindings of the Mps1 inhibitors, we performed gene silencing of Mps1 with siRNA, downregulation of the gene expression was confirmed with qPCR. We then evaluated the parameters described above confirming the decrease in cell viability upon Mps1 inhibition.

Chemical inhibition of Mps1 led to an increase in calcium in the cytoplasm, an activator of the apoptotic pathway [132], cleaved caspase 3 and PARP, $SubG_1$, Annexin V, that binds to phosphatidylserine found outside the cell membrane during apoptosis and PI staining, indicating decreased membrane integrity. In addition, decreased cell size was caused due to the shrinking of the cell, a common marker of apoptosis. This event was also observed in other cell lines with increased $subG_1$ population, decreased mitochondrial membrane potential and increased percentage of polyploid cells with no clear correlation to TP53, MYCN amplification or ALK mutational status. Therefore, these results indicate that Mps1 inhibition activates the mitochondrial and caspase-dependent apoptotic pathway.

We also evaluated other types of cell death mechanisms such as necroptosis, where traits of necrosis are also observed such as swelling of the cell, rupture of the cell

membrane and mitochondria [278]. Necroptosis was analysed by using Necrostatin-1, an inhibitor of the necrosome, a complex formed by receptor-interacting proteins needed for the activation of necroptosis [279]. The combination of Necrostatin and inhibition of Mps1, did not affect the increase in cell death in comparison to the inhibition of Mps1 alone, indicating no involvement of necroptosis in the cytotoxicity of Mps1 inhibition.

Another type of cell death is through autophagy. Autophagy is a physiological process characterised by the degradation of cytosolic components such as unfolded proteins or membranous organelles in lysosomes. Autophagy is normally used by the cell as a survival mechanism. On the other hand, excess autophagy can also lead to cell death. This type of death is known as type II programmed cell death and caspase-independent [280]. We investigated whether this death type could be induced in our experiments as previous publications presented an increase in autophagosomes by Mps1 downregulation [281]. For this evaluation, cells were stained with Acridine Orange, a permeable dye that binds to the nucleic acids and can be used to analyse autophagy due to the changes of colour from yellow to orange-red that are pH dependent. By looking at the changes in red/green ratio, no statistically significant increase in autophagy was found from Mps1 inhibition in comparison to the control.

We then proceeded to evaluate any potential changes in the cell cycle due to Mps1 inhibition, resulting in a clear disturbance in the cell cycle in comparison to the control group. Specifically, an increase in the >4N population was observed, in addition to increased cleaved DNA population confirming increased apoptosis. To evaluate more in detail these changes in the cell cycle, we stained the cells with EdU (5-ethynyl-2'-deoxyuridine), a nucleoside analogue of thymidine that can tag cells with an active DNA replication. This resulted in a reduction in the incorporation of EdU upon Mps1 inhibition.

Quantification of the levels of pH3, a marker of the mitotic phase and cycling cells, showed a reduction upon Mps1 inhibition, potentially indicating that the cells progress faster through the mitotic phase [282, 283]. On the other hand, two different pH3 positive populations were found in Mps1 inhibited cells for the 4n (diploid) and the appeared 8n (tetraploid) population.

We decided to examine more in detail the effect on mitosis by evaluating the downregulation of Mps1 in nucleus size, this resulted in a significant increase upon Mps1 silencing.

In addition, an accumulation of mitotic defaults was observed when Mps1 was either chemically inhibited or silenced with siRNA, with lack of aligned metaphase plates, asymmetric chromosome distributions and multipolar spindle organisation, therefore causing aberrant nucleus and cell division. Taken together, these results indicate that cells undergo polyploidy due to Mps1 inhibition, causing mitotic catastrophe due to defective mitosis leading to the activation of the mitotic pathway

in the daughter cells due to chromosome instability. This is shown by increased cytosolic calcium, followed by decreased mitochondrial membrane potential, caspase 3 activation, cleaved PARP and increased $SubG_1$ population.

We then evaluated the effect of Mps1 inhibition in the patient derived xenograft (PDX) model. The PDX models maintain the biological characteristics of the tissue of origin, therefore they are of high interest for the screening of drugs [284]. We used LU-NB-2, PDX cells originated from chemoresistant metastatic tumour, presenting MYCN amplification [285]. By evaluating the effect of Mps1 inhibition by Reversine or Mps-BAY2A for 72 hours on cell viability by WST-1, we could observe a decrease in cell viability in a dose-response manner. In addition, flow cytometry presented an increase in cell apoptosis evaluated by Annexin V and PI. We also observed a decrease on mitochondrial membrane potential and increase of intracellular calcium concentration. The activation of the mitochondrial apoptotic pathway was confirmed with the use of the pan-caspase inhibitor Z-VAD-fmk decreasing the significant increase on cell death observed with Mps1 inhibition. Evaluation of the cell cycle in this PDX cell line confirmed an increase in polyploidy upon Mps1 chemical inhibition.

We finally proceeded to evaluate the *in vivo* effect in a SK-N-Be2c xenografted model. Once the subcutaneous tumour was detectable by eye intraperitoneal treatment of either vehicle or Reversine was given twice a week until the tumour reached a diameter of 15mm. Caliper and weight measurements were taken twice a week. By doing so, we could observe a decrease in tumour growth in the Reversine treated group with an increase in doubling time from 4.15 to 14.29 days and no changes in body weight indicating lack of toxicity *in vivo*.

We could confirm our results with previous publications presenting the importance of Mps1 as a regulator of the spindle assembly checkpoint and the effect of its inhibition *in vitro* and *in vivo* [144, 146, 148, 281, 283, 286-294].

Conclusions Paper IV

With these data, we demonstrated the potential of Mps1 inhibition as a neuroblastoma treatment presenting increased cell death in high-risk neuroblastoma cells *in vitro* and capacity to decrease tumour growth *in vivo* in a xenografted mouse model.

Overall conclusions

- Cyclophilin inhibition presented a potent anti-fibrotic effect in liver fibrosis and a more potent anti-proliferative effect than sorafenib in liver cancer cells as well as decreasing tumour growth *in vivo*
- Cyclophilin inhibition presented a synergistic effect with the chemoagent cisplatin
- Taken together, cyclophilin inhibition could be an interesting treatment against liver fibrosis and HCC individually or in combination with other therapies
- Mps1 inhibition could be an effective neuroblastoma treatment due to its cytotoxic effect *in vitro* and capacity to decrease tumour growth *in vivo*

Future perspectives

Although the clear long-term aim of these projects is to find effective treatments against liver fibrosis, HCC and neuroblastoma, additional studies would shine a light on their effectivity and facilitate this process to benefit the patients.

In project I, we were able to show the potential of NV556 as an anti-fibrotic compound due the decrease in liver fibrosis *in vivo* and decrease collagen and LOX production. Although no changes in hepatocyte ballooning were observed a decrease in ALT and AST levels *in vivo* indicated a potential cytoprotective effect in hepatocytes, therefore a deeper understanding on the cause of this effect should be studied in the future.

CypB has been presented as an important factor for the correct folding of collagen I and III, which deficiency causes over-modification of lysyl residues leading to increased degradation [209, 210]. In our study we did not evaluate the potential effect of NV556 on collagen folding, potential studies on this effect could shine a light on the decreased secretion of procollagen type I.

In relation to cyclophilin inhibition as a treatment therapy for HCC, we also started a preliminary study on the chronic effect of NV651 in HCC cell lines. We have performed a single cell analysis by transducing HEPG2 cells using Incucyte® Cell Cycle Green/Red Lentivirus Reagent. Transduced cells incorporated Geminin-TagGFP2, a protein highly expressed in the $S/G_2/M$ phases and Cdt1-mKate2, highly expressed during G_1 phase. We evaluated NV651 effect on the cell cycle with treatment up to 120 hours using Incucyte® SX5 Live-Cell Analysis System. From 24 to 120 hours a basic analysis was performed distinguishing between G_1(red) and $S/G_2/M$ phase (green). G_1 cells transitioning to the S phase [$Yellow^+$ (green and red overlap)] were subtracted for the calculation of a pure G_1 population and late $S/G_2/M$ population.

Analysis at early time points (up to 18 hours) indicated an increase trend in late $S/G_2/M$ (low red and high green) and M/G_1 cells (low red and low green) with NV651 treatment (Figure 7A and B). On the other hand, prolonged exposure of cells with NV651 caused a high increase in the percentage of cells in the G_1 phase in comparison to the control-treated cells.

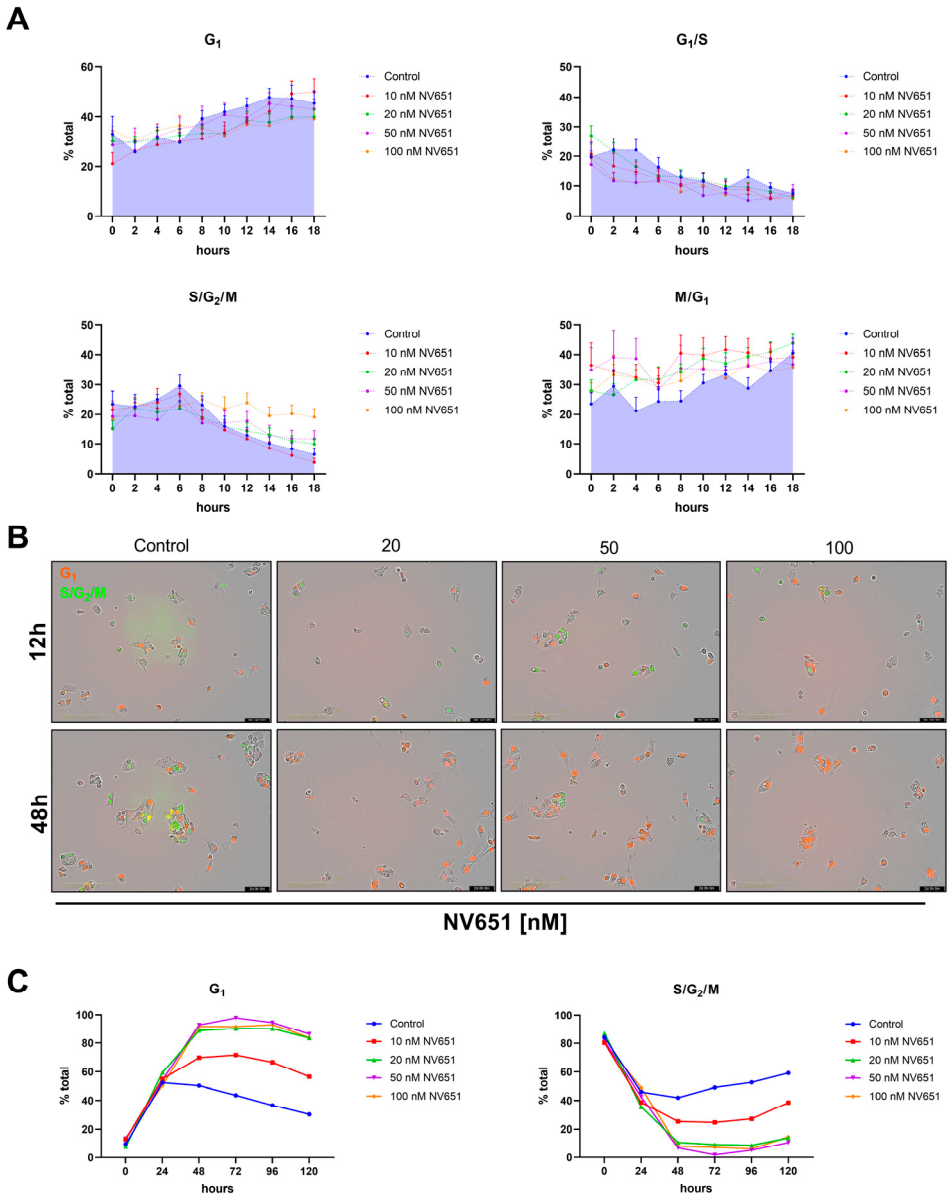

Figure 7. NV651 effect on the cell cycle. Incucyte cell-cycle analysis was performed with Incucyte Cell Cycle Green/Red transduced HEPG2 cells treated with 0, 10, 20, 50 or 100 nM of NV651 up to 120 hours. A. Percentage of cells in G_1, G_1/S, S/G_2/M or M/G_1 cells in relation to total cell number. Data is presented as mean and SEM and was calculated by adherent cell-by-cell analysis. B. Representative images of A and C analysis, where red cells are in G_1 phase and green are in S/G_2/M. C. Percentage of cells in G_1 or S/G_2/M in relation to the total cell number calculated with basic analysis.

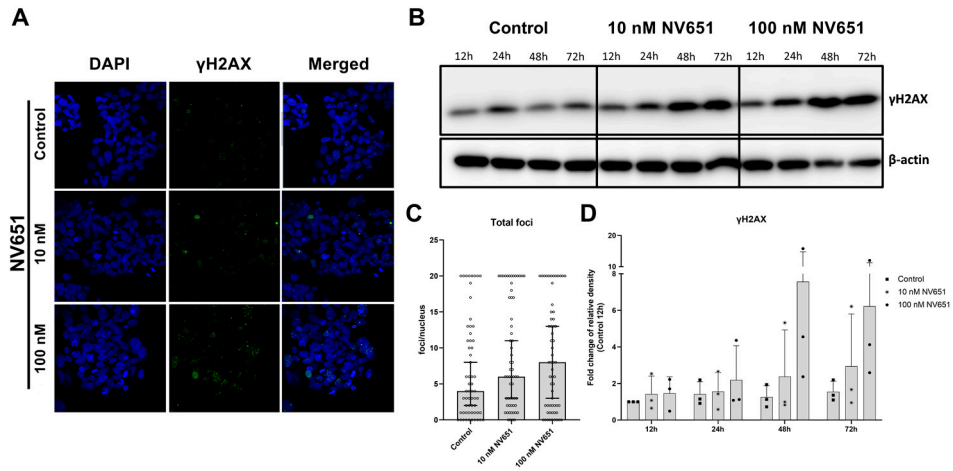

Figure 8. NV651 effect on γH2AX protein levels A. Immunofluorescence γH2AX and DAPI staining after 24 hours of 0, 10 or 100 nM of NV651 treatment. C. Number of γH2AX foci per nucleus n=1 biological replicate. B. Representative western blot of γH2AX levels in HEPG2 cells after indicated concentrations and time points. D. Quantification of γH2AX by Western Blot n=3 biological replicates. Data are presented as mean±SD

(Figure 7C). In addition, we could observe an increase trend in γH2AX, a well-known marker of DNA double-strand breaks after 24 hours of NV651 treatment (Figure 8).

Therefore, it seems that the mitotic arrest was transient and as observed in HEPG2 treated cells, after a brief mitotic arrest, cells were able to slip from mitosis and proceed to the G_1 phase for arresting. Previous publications have demonstrated that prolonged mitotic arrest can induce an accumulation of DNA damage followed by a G_1 arrest after mitotic slippage [141, 295, 296]. Indeed, evaluation of the phosphorylation levels of H2AX resulted in an increase trend after 24 hours of NV651 treatment. This hypothesis could explain the results observed with NV651 treatment, but further studies are required to confirm it.

Due to the potential of cyclophilin inhibition at different stages of the development of HCC, from NASH until the appearance of tumours, an interesting study would aim to evaluate the potential effect on a NASH model at different time points, therefore being able to observe its effect before and once the tumour is detected. Due to the effect on cancer cells and HSC, potential experiments with co-culture of HSC and HCC cell lines as mixed spheroids, HCC cell lines cultured in conditioned media by HSC or i*n vivo* HSC and HCC cells co-injected in nude mice would be of extreme interest.

In Project III, we evaluated the effect of combining both cisplatin and NV651. Potential studies for a better understanding of the cell cycle effect and confirmation of the activation of the intrinsic mitochondrial apoptotic pathway could be of great

interest. Although we could understand a bit more in detail the mechanism of action, further studies would need to be included to investigate which cyclophilin could play a bigger role in NV651 effect. In addition, silencing of the cyclophilins of interest would need to be performed to confirm the previous results.

Future experiments would need to include the evaluation of synergistic effect of NV651 with cisplatin *in vivo*. This could initially be evaluated in a HEPG2 xenografted nude mice model as the individual NV651 effect has already been confirmed in this model. Evaluation of each treatment could be done by caliper and IVIS analysis. Groups should include individual vehicle, cisplatin and NV651 and combination of the compounds to evaluate the effect on tumour growth up to 3 weeks.

Due to the common chemoresistance observed in neuroblastoma patients, with compounds such as vincristine [297, 298], future investigations in project IV will include the evaluation of treatment combinations with Mps1 inhibition and microtubule targeting compounds to overcome vincristine resistance in neuroblastoma cells.

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