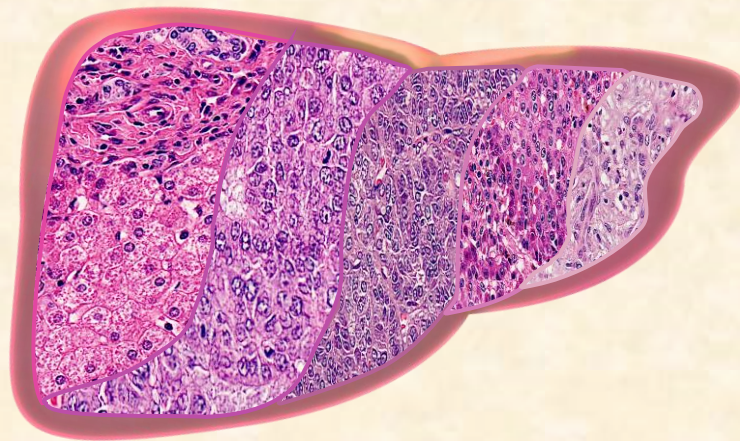# VNiVERSiDAD Ð SALAMANCA

FACULTY OF PHARMACY

Department of Physiology and Pharmacology

ROLE OF TRANSPORTOME IN CHEMORESISTANCE AND CHEMOSENSITIZATION IN HEPATOBLASTOMA

PAPEL DEL TRANSPORTOMA EN QUIMIORRESISTENCIA Y QUIMIOSENSIBILIZACIÓN EN HEPATOBLASTOMA

DOCTORAL THESIS

Candela Cives Losada

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VNiVERSiDAD Ð SALAMANCA

FACULTY OF FARMACY

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

ROLE OF TRANSPORTOME IN CHEMORESISTANCE AND CHEMOSENSITIZATION IN HEPATOBLASTOMA

Presented by **Candela Cives Losada** to obtain Ph.D. in
the University of Salamanca
In Salamanca, 25th April 2023

D. JOSÉ JUAN GARCÍA MARÍN, DIRECTOR DEL DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA DE LA UNIVERSIDAD DE SALAMANCA

CERTIFICA:

Que la Memoria titulada "**Role of transportome in chemoresistance and chemosensitization in hepatoblastoma**", presentada por Dña. Candela Cives Losada para optar al Título de Doctor por la Universidad de Salamanca, ha sido realizada bajo la dirección conjunta del Dr. D. José Juan García Marín, Catedrático del Departamento de Fisiología y Farmacología, la Dra. Dña. Rocío I. Rodríguez Macías, Catedrática del Departamento de Fisiología y Farmacología, y el Dr. D. Oscar Briz Sánchez, Profesor Contratado Doctor del Departamento de Fisiología y Farmacología de la Universidad de Salamanca.

Y para que así conste, expide y firma la presente certificación en Salamanca el día 25 de abril de dos mil veintitrés.

Fdo. José Juan García Marín

Dña. MARÍA JESÚS MONTE RÍO, COORDINADORA DEL PROGRAMA DE DOCTORADO EN FISIOPATOLOGÍA Y FARMACOLOGÍA DE LA UNIVERSIDAD DE SALAMANCA

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Fdo. María Jesús Monte Río

VI

D. JOSÉ JUAN GARCÍA MARÍN, CATEDRÁTICO DEL DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA, Dña. ROCÍO I. RODRÍGUEZ MACÍAS, CATEDRÁTICA DEL DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA, y D. OSCAR BRIZ SÁNCHEZ, PROFESOR CONTRATADO DOCTOR DEL DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA DE LA UNIVERSIDAD DE SALAMANCA
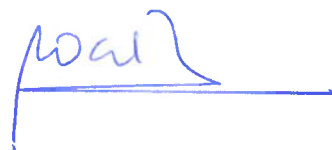
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PROYECTO EUROPEO. Comisión Europea. IMI2 Actions. 821283- TransBioLine - H2020-JTI-IMI2-2017-13-two-stage. Investigador principal: José Juan García Marín. Periodo: 2019-2024.

PROYECTO NACIONAL. Fundación La Marató de TV3. Proyecto 201916-31. Coordinador: José Carlos Fernández-Checa. Investigador principal (grupo Usal): José Juan García Marín. Periodo: 2020-2023.

PROYECTO NACIONAL. Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III. Proyectos de Investigación en Salud (AES 2019). Modalidad proyectos en salud cofinanciados con Fondos FEDER. Proyecto PI19/00189. Investigadora principal: Rocío I. Rodríguez Macías. Periodo: 2021-2023.

PROYECTO REGIONAL. Consejería de Educación, Junta de Castilla y León. Programa de apoyo a proyectos de investigación 2020. Proyecto SA074P20. Investigador principal: José Juan García Marín. Periodo: 2021-2023.

PROYECTO LOCAL. Fundación Universidad de Salamanca. PLAN TCUE 2021-2023. Prueba de concepto. Proyecto PC_TCUE21-23_011. Investigadora principal: Rocío I. Rodríguez Macías. Periodo: 2022-2023.

PROYECTO NACIONAL. Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III. Proyectos de Investigación en Salud (AES 2022). Modalidad proyectos en salud cofinanciados con Fondos FEDER. Proyecto PI22/00526. Investigador principal: José Juan García Marín. Periodo: 2023-2025.

PUBLICACIONES

Parte de los conocimientos obtenidos durante el desarrollo de esta Tesis Doctoral han sido publicados **como revisiones científicas** en las siguientes revistas:

Marin JJG, **Cives-Losada C**, Asensio M, Lozano E, Briz O, Macias RIR. Mechanisms of anticancer drug resistance in hepatoblastoma. Cancers (Basel). 2019; 11(3): 407.

Marin JJG, Monte MJ, Macias RIR, Romero MR, Herraez E, Asensio M, Ortiz-Rivero S, **Cives-Losada C**, Di Giacomo S, Gonzalez-Gallego J, Mauriz JL, Efferth T, Briz O. Expression of chemoresistance-associated ABC proteins in hepatobiliary, pancreatic and gastrointestinal cancers. Cancers (Basel). 2022; 14(14): 3524.

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Marin JJG, Macias RIR, **Cives-Losada C**, Peleteiro-Vigil A, Herraez E, Lozano E. Plasma membrane transporters as biomarkers and molecular targets in cholangiocarcinoma. Cells. 2020; 9(2): 498.

Marin JJG, Macias RIR, Monte MJ, Romero MR, Asensio M, Sanchez-Martin A, **Cives-Losada C**, Temprano AG, Espinosa-Escudero R, Reviejo M, Bohorquez LH, Briz O. Molecular bases of drug resistance in hepatocellular carcinoma. Cancers (Basel). 2020; 12(6): 1663.

Marin JJG, Perez-Silva L, Macias RIR, Asensio M, Peleteiro-Vigil A, Sanchez-Martin A, **Cives-Losada C**, Sanchon-Sanchez P, Sanchez De Blas B, Herraez E, Briz O, Lozano E. Molecular bases of mechanisms accounting for drug resistance in gastric adenocarcinoma. Cancers (Basel). 2020; 12(8): 2116.

Marin JJG, Sanchon-Sanchez P, **Cives-Losada C**, Del Carmen S, González-Santiago JM, Monte MJ, Macias RIR. Novel pharmacological options in the treatment of cholangiocarcinoma: mechanisms of resistance. Cancers (Basel). 2021; 13(10): 2358.

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Comunicaciones a congresos

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Asensio M; Lozano E; **Cives-Losada C**; Carrillo J; Abete L; Briz O; Cairo S; Bruiz J; Armengol C; Marin JJG; Macias RIR. "Role of drug transporters in the chemoresistance of hepatoblastoma". International Liver Congress. European Association for the study of the liver (EASL). Viena (Austria). 2019. Tipo de comunicación: póster.

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Cives-Losada C; Briz O; Cairo S; Efferth T; Martínez-Chantar ML; Avila MA; Armengol C; Macias RIR; Lozano E; Marin JJG. "Investigation of novel hepatoblastoma chemosensitizers based on the inhibition of ABC pumps-mediated drug efflux". 4th

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Cives-Losada C; Briz O; Cairo S; Efferth T; Martínez-Chantar ML; Avila MA; Armengol C; Macias RIR; Lozano E; Marin JJG. " Nuevos inhibidores de las bombas ABC como agentes sensibilizantes del hepatoblastoma al tratamiento farmacológico". 48º Congreso Anual de la Asociación Española para el Estudio del Hígado (AEEH). Madrid (España). 2023. Tipo de comunicación: póster.

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La doctoranda ha participado en otros proyectos de los que se han derivado, entre otras, las siguientes participaciones en congresos:

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Espinosa-Escudero R; Alonso-Pena M; Herraez E; Monte MJ; Briz O; Sanchez-Martin M; Garcia-Tunon I; Berrocal P; **Cives-Losada C**; Sanchez de Blas B; Prieto J; Marin JJG. "Toxicidad del ácido trihidroxicolestanoico (THCA) en modelos *in vitro* e *in vivo* de deficiencia parcial de acil-coaoxidasa 2 (ACOX2)". XIV Jornadas Científicas del CIBERehd. *Online*. 2020. Tipo de comunicación: póster.

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extracorpórea no invasiva de la función hepatobiliar utilizando nuevos derivados de ácidos biliares con fluorescencia en el infrarrojo cercano". 3ª Reunión de Hepatología Traslacional. Alicante (España). 2021. Tipo de comunicación: póster.

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Lozano E; Arretxe E; Lapitz A; Bustamante J; Boix L; **Cives-Losada C**; Izquierdo-Sanchez L; Alonso C; Gonzalez LM; Gonzalez-Santiago J; Salvador P; Bruix J; Bujanda L; Muñoz-Bellvis L; Banales JM; Marin JJG; Macias RIR. "Usefulness of serum metabolomic profiling for the differential diagnosis in liver cancer: a validation study". EASL Digital Liver Cancer Summit. *Online.* 2022. Tipo de comunicación: póster.

Romero MR; Temprano AG; Sanchez de Blas B; Espinosa-Escudero R; **Cives-Losada C**; Lozano E; Briz O; Monte MJ; Perez-Melero C; Bermejo F; Marin JJG. "Determinación extracorpórea no invasiva de la función hepatobiliar utilizando nuevos derivados de ácidos biliares con fluorescencia en el infrarrojo cercano". 47º Congreso Anual de la Asociación Española para el Estudio del Hígado (AEEH). Madrid (España). 2022. Tipo de comunicación: póster.

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ABREVIATIONS

2D	Two-dimensional
5-FU	5-Fluorouracil
ABC	ATP-binding cassette
AFP	α-Fetoprotein
BCRP	Breast cancer resistance protein
bMRP1	Bovine MRP1
CAM	Calcein acetoxymethylester
CCA	Cholangiocarcinoma
cDNA	Complementary DNA
CF	Carboxyfluorescein diacetate
CHIC	Children's Hepatic Tumors International Collaboration
COG	Children's Oncology Group (USA)
CR	Cisplatin-resistant
CRYO	Caryophyllene oxide
CTR1	Copper transporter 1
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DR	Doxorubicin-resistant
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
FTC	Fumitremorgin C
GLUT1	Glucose transporter 1
GPOH	German Pediatric Oncology and Hematology
GSH	Reduced glutathione
HB	Hepatoblastoma
HCC	Hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hh	Hedgehog
hMRP1	Human MRP1
HRP	Horseraddish peroxidase
HTA	Human Transcriptomic Array
IC$_{50}$	Inhibitory concentration 50
IF	Immunofluorescence
IGF	Insulin-like growth factor
IGTP	Germans Trias i Pujol Institute
IHC	Immunohistochemistry

INI1	Integrase interactor 1
JPLT	Japanese Study Group for Pediatric Liver Tumors
kDa	kiloDalton
LAT1	L-type amino acid transporter 1
MATE	Multidrug and toxin extrusion transporter
MDR	Multidrug resistance
MDR1	Multidrug resistant protein 1
MFS	Major Facilitator Superfamily
MOC	Mechanism of chemoresistance
MRP	Multidrug resistant-associated protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Mitoxantrone
NBD	Nucleotide-binding domain
NR	Non-responder (to treatment)
NT	Non-tumor
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OCTN	Organic cation/carnitine transporter
PBS	Phosphate buffered saline
PDB	Protein Data Bank
PDX	Patient-derived xenograft
PEPT	Peptide transporter
PHITT	Pediatric Hepatic International Tumor Trial
PI	Propidium iodide
POSTTEXT	POSTTreatment EXTent of disease
PRETEXT	PRETreatment EXTension of the tumor
qPCR	Quantitative PCR
R	Responder (to treatment)
Rho	Rhodamine 123
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RT	Reverse transcription
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SIOPEL	International Childhood Liver Tumors Strategy Group
SLC	Solute Carrier
SNP	Single nucleotide polymorphism

T	Tumor
TACE	Transarterial chemoembolization
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TKI	Tyrosine kinase inhibitor
TLDA	TaqMan Low Density Array
TMD	Transmembrane domain
Tris-HCl	Trishydroxymethyl aminomethane hydrochloride
Trizma	Trishydroxymethyl aminomethane base
TXP	Triple X proteomics
UPLC	Ultra-performance liquid chromatography
WB	Western blot
WT	Wild type
YAP	YES-associated protein
ZIP	Zero interaction potency
β-hCG	β-human chorionic gonadotropin
λ	Wavelength

PRETEXT annotations:

C	Infiltration of the caudate lobe
E	Extrahepatic spread of disease
F	Multifocality
M	Distant metastases
N	Lymph node metastases
P	Invasion of the portal vein
R	Tumor rupture or intraperitoneal bleed
V	Involvement of hepatic veins

INDEX

1. AIMS

Hepatoblastoma (HB) is the most frequent pediatric liver cancer. Despite its incidence is low (2 children per million per year) this has increased over the last few years. Surgical resection, liver transplantation and chemotherapy based mainly on cisplatin and doxorubicin are the current options for treating these patients. Unfortunately, 20% of patients still have very poor prognosis mainly due to the lack of response to treatment. Moreover, 12% of patients in complete remission are likely to relapse.

This refractoriness may be due to the presence of a multidrug resistance (MDR) phenotype in cancer cells, conferred by complex and powerful mechanisms of chemoresistance (MOCs). These may be intrinsically present in tumor cells or may be acquired during treatment. Our research group has classified MOCs into seven groups.

In this thesis, we have focused on studying in depth one of them, MOC-1, which refers to the reduction of the amount of intracellular drug due to decreased expression and/or function of plasma membrane solute carriers (SLCs) involved in drug uptake (MOC-1a) or increased expression and/or function of transporters of the ATP-binding cassette (ABC) superfamily of proteins involved in drug export (MOC-1b).

The identification of plasma membrane transporters responsible for anticancer drug refractoriness in HB could be extremely useful as diagnostic and prognostic biomarkers or as targets for designing chemosensitization strategies.

Given this background, the **overall aim** of this PhD thesis was to characterize the role of the transportome in the lack of response of HB to chemotherapy and subsequently design pharmacological strategies to overcome HB chemoresistance.

To achieve this goal, we defined several partial aims.

A) To determine the role of the transportome among other mechanisms of HB chemoresistance we stablished the following aims:

1) To identify drug transporters involved in the lack of response of HB to pharmacological treatment.

2) To characterize the transportome in *in vitro* experimental models of HB, using both classical and patient-derived xenograft cell lines.

3) To study the effect of doxorubicin and cisplatin on the transportome in HB cells, after both short-term and long-term exposure to pharmacological stress.

4) To investigate cross-resistance to drugs used in second-line treatment of HB using chemoresistant cell lines generated for this purpose.

B) To develop HB chemosensitization strategies we proposed:

5) To evaluate strategies to improve response to doxorubicin based on compounds with the ability to inhibit MDR1 in HB cells, both *in vitro* and *in vivo*.

6) To search *in silico* for MRP1 and MRP2 inhibitors through a molecular docking study and to evaluate *in vitro* the chemosensitizing effect of the selected compounds to doxorubicin and cisplatin.
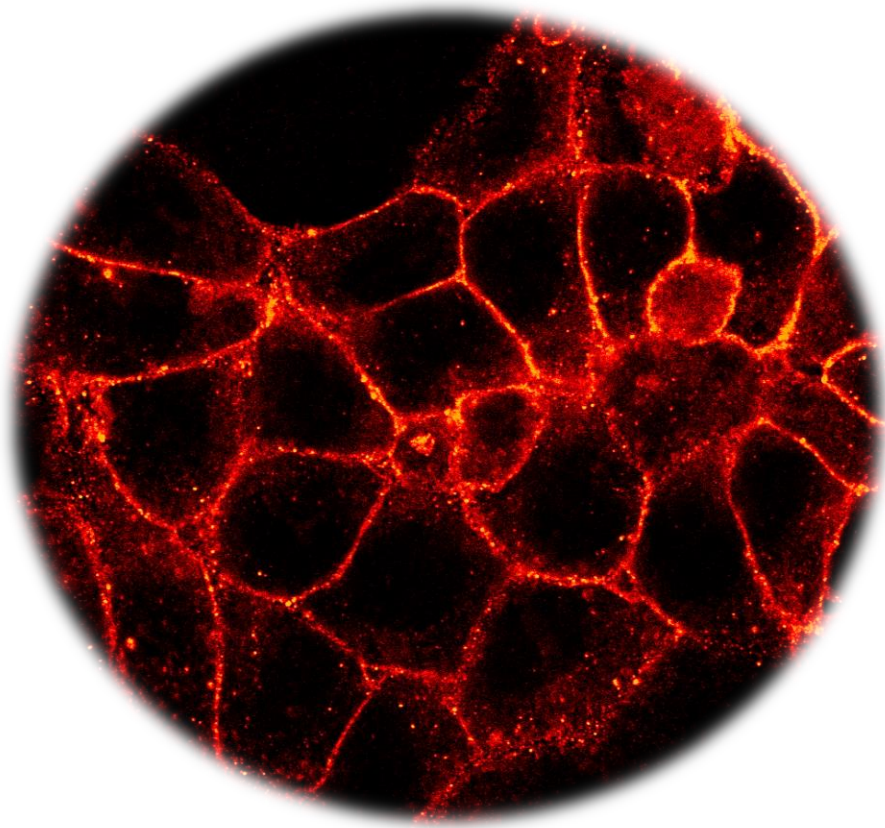
2. INTRODUCTION

2.1. HEPATOBLASTOMA

2.1.1. Epidemiology

Hepatoblastoma (HB) is a malignant tumor of embryonal origin that develops in the absence of underlying liver diseases [1]. Although HB is a rare tumor, affecting approximately 2 children per million, it is the most common childhood liver cancer, accounting for about 90% of malignant liver tumors in children under 4 years of age [2, 3]. Its incidence has increased worldwide more than that of other pediatric cancers for the last 20 years [4, 5]. HB appears more frequently in male than female children, with a ratio of 1.5:1 to 2:1 [4].

2.1.2. Etiopathogenesis

Several studies have attempted to identify somatic mutations associated with carcinogenesis in HB. However, the frequency of oncogenic variants is very low, the smallest among pediatric tumors [6], with an average of 2.9 mutations per tumor, which is lower than that in hepatocellular carcinoma (HCC) in adults [7-10] (**Figure I1**).
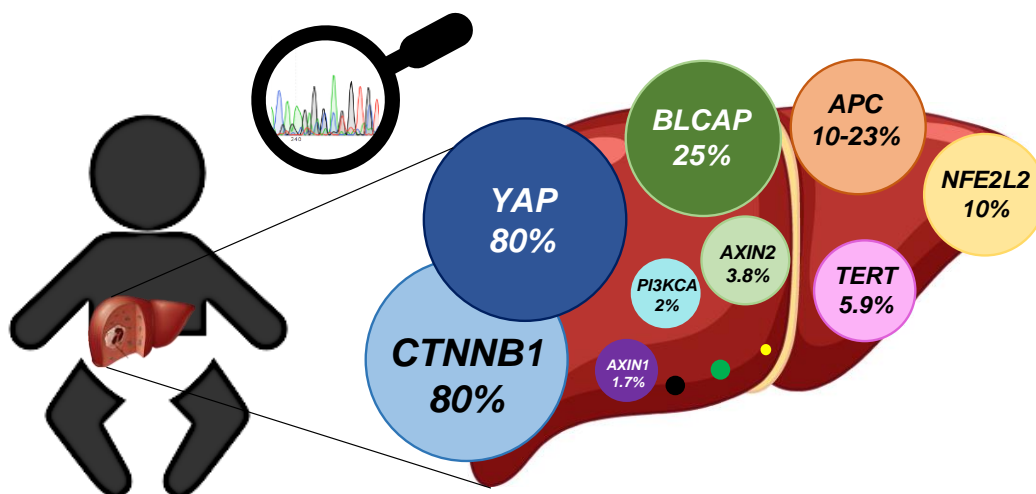
Figure I1. Most common genetic and epigenetic alterations in hepatoblastoma. Gene and frequency of detection (% of cases). Information obtained from [11].

HB has been associated, especially, with constitutive activation of the Wnt/β-catenin signaling pathway [12, 13], involved in liver development, regeneration, and metabolic zonation, which is dysregulated in several types of cancers [14]. Furthermore, strong nuclear immunoreactivity for c-MYC, a target of the Wnt/β-catenin pathway, was detected in 83% of HB samples (10 of 12) in the study carried out by Ranganathan *et al.* [15]. In 70% of cases this alteration is due to the presence of mutations in the *CTNNB1*

gene, which encodes the β-catenin protein [8, 10, 16, 17]. In a lower number of cases, this overactivation is caused by inactivating mutations in *AXIN1*, *AXIN2* [18], and *APC* (the latter in patients with familial adenomatous polyposis) [19], encoding proteins that are part of the ß-catenin degradation complex.

Around 50-60% of HBs show dysregulation of the Hippo pathway, which mainly results in the inability to phosphorylate and degrade YES-associated protein (YAP), the main downstream effector of this pathway [6]. This protein is a transcriptional coactivator that regulates tissue growth, organ size and stem cell maintenance. Notably, concomitant mutations of YAP and β-catenin have been found in 80% of cases and overactivation of both genes results in HB development in animal models [20-22].

In addition, high activity of the Hedgehog (Hh) pathway, crucial for the growth and differentiation of many tissues throughout embryonic development, has been detected in HB. This has been associated with an elevated expression of Shh, GLI1 and PTCH1 or silencing of the promoter of the Hedgehog interacting protein (HHIP) gene – a tumor suppressor that inactivates components of the Hedgehog cascade [11, 23].

The frequency of mutations of *NFE2L2*, the gene encoding NRF2, in HB samples is 7-10% [8, 24, 25]. These mutations prevent KEAP1-mediated degradation, activating the NRF2-KEAP1 pathway, which regulates cytoprotective responses to endogenous and exogenous stress caused by reactive oxygen species [11]. Somatic mutations have been identified in other oxidative stress regulatory genes, such as inactivating mutations in *TXNDC15* and *TXNDC16* genes [24].

The PI3K/AKT/mTOR pathway, which regulates cell proliferation, survival, motility, and metabolism, is also altered in HB. For instance, gain-of-function mutations of the p110α subunit of PI3K (PI3KCA) have been detected in 2% of HB cases [26].

Insulin-like growth factor (IGF) signaling may be also involved in HB development [27]. In this case, the aberrant signal is induced by IGF2 ligand overexpression, as well as reduced levels of inhibitors, *i.e.*, IGF-binding proteins 2 and 3 (IGFBP2 and IGFBP3) [28, 29].

Dysregulation of other pathways involved in cell growth and survival that can participate in carcinogenesis have also been described in HB [30], as well as factors that regulate energy metabolism, such as FOXA3 [31].

Mutations were also found in the reverse telomerase promoter (TERT), as well as the presence of chromosomal instability due to deletions of tumor suppressor genes such

as *RAD17* and *TP53* [8, 11]. A whole exome sequencing analysis of HB revealed mutations in other genes such as *ERBB4*, *MDM4*, *FBXW7*, *SRC* and *BRCA2*, with a role in cell cycle control [32].

Interestingly, around 25% of HB present alterations in RNA editing of *BLCAP* gene, which encodes a protein that reduces cell growth by stimulating apoptosis [33].

No clear association between the development of HB in children and the presence of environmental factors such as smoking or alcohol consumption before or during pregnancy, maternal illness, or medication during pregnancy has been found [34, 35]. However, the occurrence of this type of tumor has been correlated with low birth weight (less than 1.5 kg) [36, 37]. This could be due to iatrogenic damage, as preterm infants are often exposed to potentially toxic environmental agents during their stay in neonatal intensive care units (radiation, transfusions, drugs, etc.), at a time when fetal tissues are particularly sensitive due to their immaturity [38, 39]. Children born after infertility treatments seem to have more risk of developing HB [4]. Preeclampsia, poly/oligo-hydramnios, and high pregnancy weight could also favor HB development [37].

An inherited predisposition to develop HB has been described in a low proportion of cases (less than 10%) [40] in patients with some rare disorders, such as Aicardi [41], Simpson-Golabi-Behmel [42], familial adenomatous polyposis [19, 43], Beckwith-Wiedemann, and trisomy 18 [3] syndromes.

2.1.3. Classification

Although the etiology of HB is not well established, it is thought to be due to altered developmental processes before the differentiation of hepatoblasts into hepatocytes is complete. This concept is supported by the fact that the tumors are formed by immature cells and are often detected in children under one year of age or even before birth [1, 44].

At the histopathological level, HBs encompass different stages of liver development, including epithelial, mesenchymal, or mixed forms [12, 45] (**Table I1**).

Table I1. Histologic classification of hepatoblastoma, extracted from [6].

Type	Subtype and/or Properties
Epithelial	
Fetal	• <u>Well-differentiated</u>: uniform with round nuclei, cords with minimal mitotic activity; may be associated with EMH. • <u>Crowded</u>: mitotically active, prominent nucleoli. • <u>Pleomorphic</u>: poorly differentiated, high nuclear:cytoplasmic ratio; moderate anisonucleosis. • <u>Anaplastic</u>: marked nuclear enlargement, pleomorphism and hyperchromosia; abnormal mitoses.
Embryonal	Smaller cells; high nuclear:cytoplasmic ratio; primitive tubules may be associated with EMH.
Macrotrabecular	Fetal or embryonal type growing in clusters of 5 or more cells between sinusoids.
Small cell undifferentiated	About half the size of fetal HB; minimal pale & amphophilic cytoplasm; oval/round nuclei; fine chromatin; inconspicuous nucleoli.
Cholangioblastic	Presence of bile ducts that may be quite prominent.
Mixed	
Stromal derivative	Spindle cells/blastema; presence of other components (osteoid, cartilage, muscle).
Teratoid	Mixed; primitive endoderm; presence of neural derivatives; squamous and/or glandular components, melanin.

EMH: extramedullary hematopoiesis.

Well-differentiated fetal tumors have a fully epithelial phenotype, whereas poorly differentiated tumors are characterized by a predominantly mesenchymal component and a higher rate of mitotic cells [45]. HBs also differ in gene expression pattern, prognosis, and response to chemotherapy [46]. Long-term survival is poorer for patients with undifferentiated HB [47]. These HBs, especially the small cell ones, have a worse prognosis and a poorer response to chemotherapy [48]. Furthermore, the acquisition of mesenchymal characteristics is associated with more aggressive invasion, metastasis, and chemoresistance [49]. Epithelial tumors include several subtypes such as pure fetal with varying degrees of mitotic activity, embryonal, pleomorphic, macrotrabecular, undifferentiated small cell, cholangioblastic, and mixed subtypes [12] (**Figure I2**).
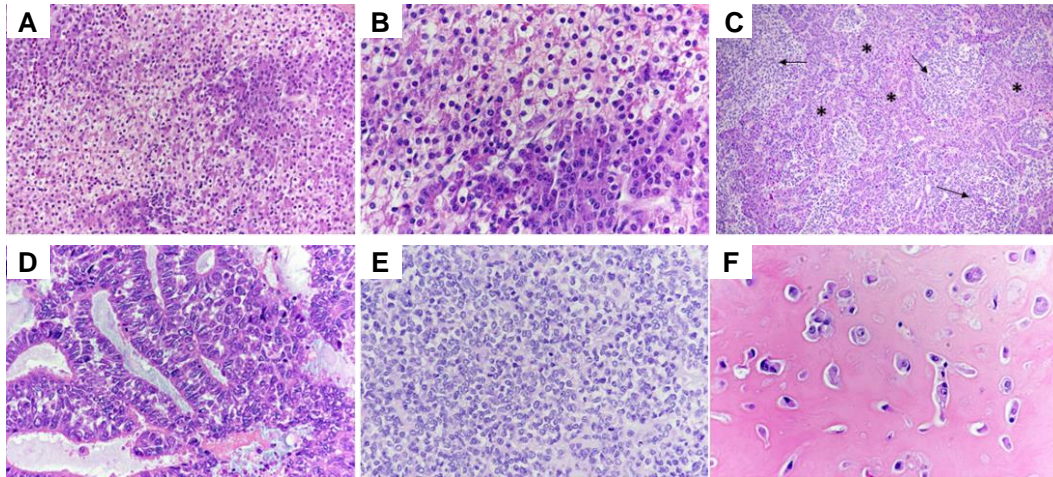
Figure I2. Representative images of the different histological hepatoblastoma subtypes stained with hematoxylin and eosin. Pure fetal HB showing the organization in trabeculae of the tumor (**A** in 200x and **B** in 400x). Mixed HB (**C** in 100x), where asterisks indicate fetal tumor cells and arrows embryonal HB cells. Rosettes ("pseudo-rosettes"), acini, tubules in embryonal-like HB cells (**D**, in 400x). Small cell undifferentiated HB (**E** in 400x). Mixed epithelial-mesenchymal HB containing osteoid as a mesenchymal component (**F** in 400x). Extracted from [12].

Results from studies of genomic alterations, transcriptomic studies such as microarrays or RNASeq, epigenetic or metabolomic analyses have been used to establish molecular classifications of HB [33, 49-51].

First, Cairo *et al.* identified two subtypes (C1 and C2) of HB, based on the expression profile of 16 genes. Specifically, the C1 group showed overexpression of mature hepatocyte markers (such as *GLUL, RHBG* and *CYP2E1*), and resembled the molecular characteristics of fetal liver. In contrast, subtype C2 was mainly comprised of tumors with an embryonal phenotype, showing increased liver stem/progenitor cell and proliferation markers (such as *AFP, IGF2* or *BIRC5*) and activation of c-MYC signaling. This subtype also showed elevated invasive and metastatic characteristics [50, 52]. C1/C2 classification was also associated with histological subtypes, and it was found that C1 tumors showed mainly a mitotically inactive fetal phenotype, while C2 tumors had a more immature pattern including predominance of fetal, macrotrabecular and embryonal components [50]. In turn, Hooks *et al.* proposed a subclassification of the C2 group into two distinct subgroups, C2A and C2B, based on the expression of four genes (*HSD17B6, ITGA6, TOP2A* and *VIM*). In particular, the C2A group displayed a highly proliferative and aggressive phenotype with high *TOP2A* expression and activated DNA repair pathway, while the C2B group exhibited a mesenchymal phenotype with spindle-shaped structures and is characterized by high *VIM* expression [49].

Carrillo-Reixach *et al.* described two subtypes of HB, called epigenetic clusters A and B (Epi-CA and Epi-CB), based on the differences in the degree of DNA and CpG island hypomethylation in the tumor cells [33], which were also strongly associated with the molecular subclasses C1/C2A/C2B [33, 49]. Other studies have focused on identifying the methylation patterns of tumor suppressor genes to identify those that determine the malignancy of HB [53].

A combination of genomic and transcriptomic analysis was used by Sumazin *et al.* to identify diagnostic, therapeutic and prognostic biomarkers. These allowed them to distinguish three molecular subtypes (HB1, HB2 and HB3) based on prognosis. Candidate biomarkers for their predictive capacity include *HNF1A, NFE2L2, SALL4, HMGA2* and *LIN28B* [54].

Finally, Crippa *et al.* characterized the metabolic profile of HB and HCC cell lines and observed that expression of numerous glycolytic enzymes, such as *HK1*, *PFKP* and *LDHB*, were elevated in cells with an embryonic phenotype compared to fetal-type HB cells, which showed higher levels of gluconeogenesis enzymes, such as *PPARGC1A*, *AQP9*, *GK* and *G6PC* [51].

2.1.4. Diagnosis

The tumor is usually detected during the child's three first years of life, most frequently between 3-4 years, although it is also common to diagnose HB during fetal development. Congenital HB is diagnosed *in utero* or in the first month of life [40, 55].

Neonatal HB usually presents as a palpable abdominal mass, increased abdominal distension resulting in respiratory distress, and hepatomegaly [40]. Weight loss and thriving difficulties are also signs of tumor presentation [11]. Patients may also have a marked thrombocytosis, which could be due to enhanced thrombopoietin production in tumor tissues [40].

α-fetoprotein (AFP) is commonly used as a marker for screening and diagnosis of this tumor, as it is elevated in approximately 90% of patients; however, it is neither sensitive nor specific for HB [40]. β-human chorionic gonadotropin (β-hCG) is increased in approximately 20% of patients [56]. The secretion of this hormone produced virilization and isosexual precocity in some cases of boys under 3 years of age [40].

On ultrasound, HB appears as a well-defined echogenic mass. Areas of necrosis, hemorrhage and calcifications may be seen within the neoplasm. Computed tomography

(CT) and magnetic resonance imaging (MRI) scans are used to identify vessel involvement and distant metastases [11].

After physical examination, biochemical markers and imaging, histological diagnosis is required, although some experts believe that biopsy may not be necessary for young children (6 months to 3 years) with an extremely high AFP level [56]. The biopsy material is usually obtained percutaneously, although fine needle aspiration has also been proposed [57, 58]. This tumor sample will be used to confirm the diagnosis and to elucidate the histopathological type [40]. For example, the immunohistochemical expression of integrase interactor 1 (INI1, coded by *SMARCB1*) in the small cell undifferentiated subtype is relevant in the differential diagnosis of malignant rhabdoid tumors and HB [59, 60], while SALL4 can be used to distinguish the embryonal subtype [61].

Minimally invasive methods based on liquid biopsy are being developed to detect circulating materials in the patients' blood. Different markers (tumor cells, DNA, RNA) detected in the sample can be useful for diagnosis, prognosis, and monitoring the disease [62]. As an example, it has been proposed that exosomal miR-21 could be employed to diagnose and to predict prognosis in patients with HB [63], although the results need to be validated to reach the clinic.

2.1.5. Treatment

The International Childhood Liver Tumors Strategy Group (SIOPEL) distinguished between two risk groups based on tumor invasion and metastases or ruptured tumors: standard-risk and high-risk [64, 65]. Later, SIOPEL also considered AFP levels, establishing an association with poor prognosis if the serum values are lower than 100 ng/ml or higher than 1.2×10^6 ng/ml [66].

A system called PRETEXT (PRETreatment EXTension of the tumor) has been stablished to assess the extent of the tumor within the liver before treatment, which allows stratification of newly diagnosed patients to select the most appropriate treatment strategy for each patient [65]. Thus, HB are classified into four PRETEXT groups (I-IV), depending on the affected liver surgical section [55, 67] (**Figure I3**). The hepatic veins (right and middle) and umbilical fissure define the boundaries of each section [68]. There are additional factors that are studied to further describe the tumor: involvement of hepatic veins (V), invasion of the portal vein (P), extrahepatic spread of disease – it crosses tissue boundaries (E), infiltration of the caudate lobe (C), tumor rupture or

intraperitoneal bleed (R), lymph node metastases (N), multifocality (F); and presence of distant metastases (M) [5, 55, 69].

SECTION	PRETEXT I	PRETEXT II	PRETEXT III	PRETEXT IV
N. involved	1	1-2	2-3	4
N. adjoining tumor-free	3	2	1	-
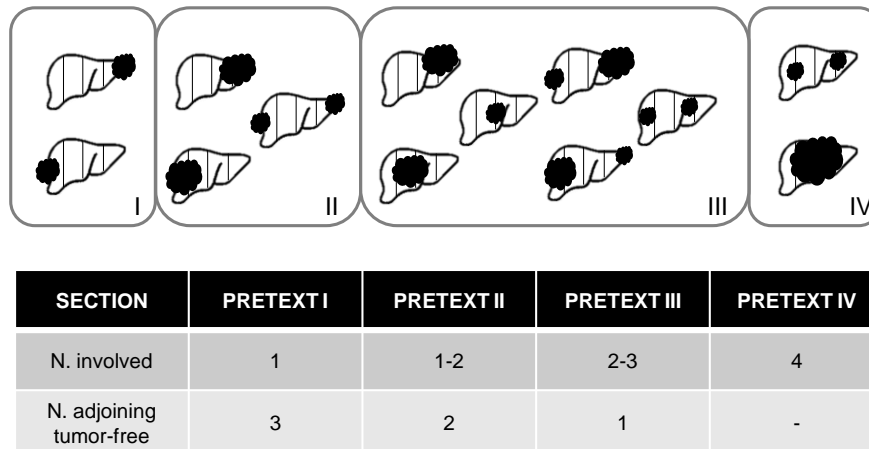
Figure I3. Characteristics of PRETEXT groups (I-IV). Modified from [5]. N, number; PRETEXT, PRETreatment EXTent of disease.

The major cooperative groups - SIOPEL, USA's Children's Oncology Group (COG), the German Society for Pediatric Oncology and Hematology (GPOH), and the Japanese Study Group for Pediatric Liver Tumors (JPLT) - joined forces and funded the Children's Hepatic Tumors International Collaboration (CHIC). CHIC proposed an additional classification to evaluate the risk (CHIC's Hepatoblastoma Stratification, CHIC-HS) based on data from 1605 patients considering their age, AFP levels and PRETEXT classification and annotation. CHIC-HS distinguished among very low-, low-, medium-, and high-risk HB. If surgical resection at diagnosis is feasible, the HB is considered very low risk [70] (**Figure I4**). This classification was considered for the patients enrolled in the Pediatric Hepatic International Tumor Trial (PHITT, NCT03017326), which described the pathology and aims to improve treatment protocols based on the tumor's risk [70]. The results will shine a light on the optimal dosage of preoperative chemotherapy, its role after liver transplantation and the management of patients who relapse [71].

Currently, there are three different options to treat HB: i) pre- and/or post-operative (neo-adjuvant and/or adjuvant) chemotherapy, ii) surgical procedure for tumor resection, and iii) liver transplantation [55, 72, 73]. The therapeutic approach for patients with HB differs between international collaborative groups. For example, tumor resection for localized HB is followed by chemotherapy in the United States, whereas in Europe, neoadjuvant chemotherapy is recommended prior to surgery [74, 75]. Nowadays, neoadjuvant chemotherapy is always recommended for advanced stages [76].
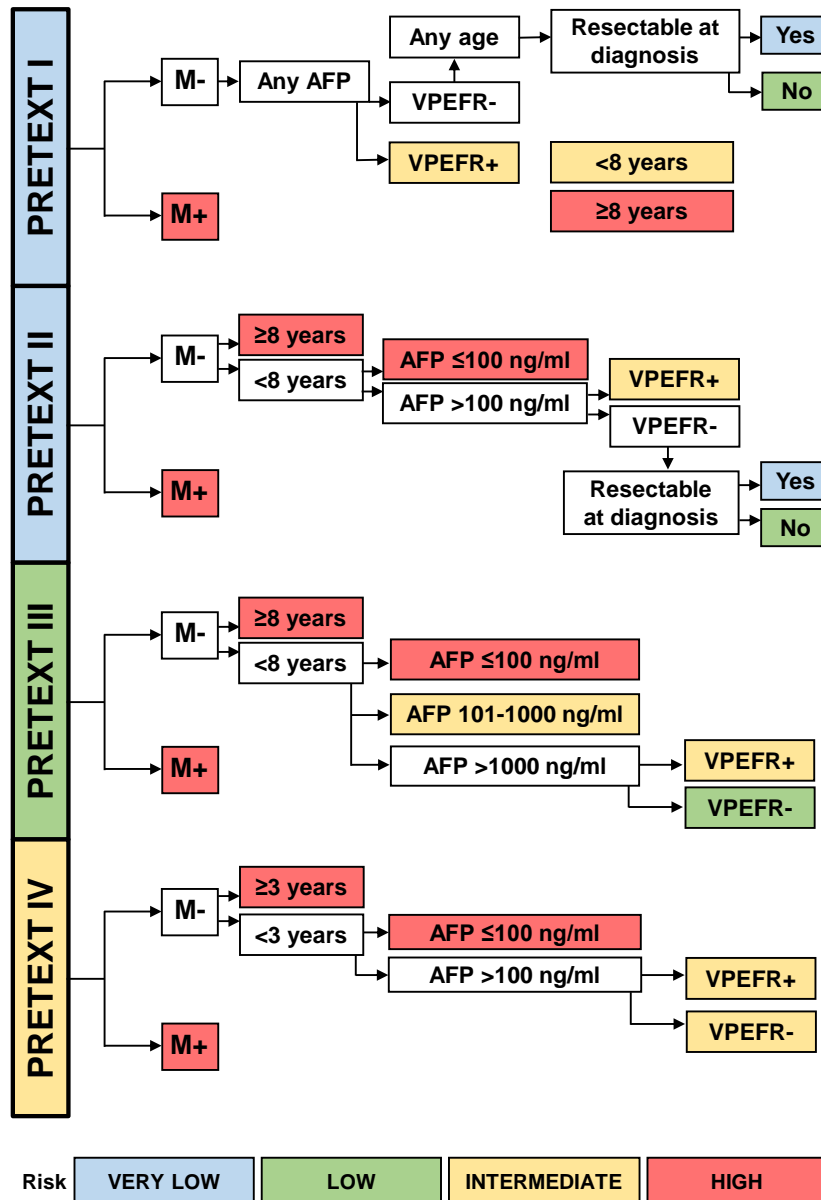
Figure I4. Risk stratification trees for each PRETEXT group by the Children's Hepatic Tumors International Collaboration (CHIC). AFP, α-fetoprotein; E, extrahepatic disease; F, multifocality; M, metastases; P, invasion of the portal vein; PRETEXT, PRETreatment EXTent of disease; R, tumor rupture; V, cava/hepatic veins involvement. The color of the parameter indicates the risk state (high, red; intermediate, yellow; low, green, and blue, very low). Modified from [70].

The new stratification system, considering PRETEXT stages, age, and AFP levels, is key to design a suitable treatment plan adapted to the patient's risk [67, 77-79]. Primary resection of HB is recommended to treat well-differentiated HB at an early stage [80, 81], later designated as very low risk tumor **(Figure I4)** [82].

Neoadjuvant pharmacological therapy is often needed to reduce tumor size and perioperative morbidity [82, 83]. All chemotherapeutic regimens to treat HB contain cisplatin. In the SIOPEL-2, -3 and -4 trials, when the first risk stratification (standard *vs*

high) was applicable, neoadjuvant treatment consisted of cisplatin alone for the first group of patients, while a combination of cisplatin and doxorubicin (PLADO) was administered to the second one [65, 84]. On the other hand, COG recommended its combination with 5-fluorouracil (5-FU) and vincristine (C5V) and the addition of doxorubicin (C5VD) depending on the patient's risk [81, 85].

One of the aims of the SIOPEL trials was to adjust the dosage of the antitumor agents, to reduce the adverse effects in treated children [65]. Cisplatin can cause nephrotoxicity and ototoxity [86], whereas doxorubicin can induce cardiotoxicity [65]. Sodium thiosulfate has been administered after cisplatin in the SIOPEL-6 trial, which reduced hearing loss in standard-risk HB patients [87]. Radiation can be considered for patients who do not tolerate systemic chemotherapy along with transarterial chemoembolization (TACE) [5].

To monitor the effect of neoadjuvant chemotherapy before surgery, the tumors are examined by cross-sectional imaging [73] and AFP serum levels are determined [88, 89]. Then, HB can be classified with the POSTTEXT (POSTTreatment EXTent of disease after chemotherapy) considering i) resection at diagnosis (complete or with residual microscopic disease), ii) biopsy without resection at diagnosis and iii) distant metastases [5, 69, 90].

When the patients do not respond to chemotherapy due to resistance to administered drugs, the period of treatment can be extended [55]. However, prolonged chemotherapy seems ineffective [81]. Other antitumor drugs can be used in patients with advanced and resistant HB, such as anthracyclines (epirubicin or idarubicin), platinum derivatives (carboplatin or oxaliplatin), irinotecan, 5-FU, nitrogen mustards (ifosfamide or cyclophosphamide) and *Vinca* alkaloids (vincristine or vinblastine) [91].

Although some tyrosine kinase inhibitors (TKIs) such as sorafenib, regorafenib, lenvatinib, and cabozantinib are drugs of choice for the treatment of HCC, both in adults [92] and children [93], their use in the treatment of HB is very limited. However, its use in patients with recurrent metastatic HB has achieved good results [94]. Based on the paucity of other therapeutic options, the use of combinations with TKIs should be considered as therapy in patients with recurrent HB who have not responded to traditional agents. In addition, these drugs have been shown to have a potent antiproliferative effect on HB cell lines and in patient-derived xenograft (PDX) models implanted in mice [95, 96].

If neoadjuvant chemotherapy treatment works, the tumor will be surgically resected [90], but if the conditions did not improve, the patient will be proposed for liver transplant [97,

98]. Indeed, this is recommended for patients with nonresectable HB [88] because of their multifocality and/or vascular invasion (in PRETEXT IV tumors), because of the involvement of the cava/hepatic veins (in PRETEXT III tumors) when tumors closely surround or enclose large vessels or because of their lack of response to chemotherapy [99, 100]. Better results are obtained with a living donation and within the first two years of life due to the immaturity of the immune system [101].

Chemotherapy and advances in surgical equipment and techniques have provided an improvement in survival rates (70-80%) [4, 65]. In fact, most HB patients respond to standard treatment with a significant reduction in tumor size [102], but around 20% of cases presenting with more aggressive chemoresistant tumors have very poor prognosis [81, 103, 104]. Besides, around 12% of patients in complete remission are likely to relapse [105].

CHIC has reported a correlation between some factors and the patients' prognosis [78]. For example, worse prognosis was observed in patients with tumor size >5 cm [106], older age at diagnosis [107]; AFP levels <100 ng/ml or >1x10^6 ng/ml, PRETEXT IV and VEF+ tumors, presence of metastases [5, 108], lung metastases developed during treatment [109] or tumor rupture at diagnosis. Small cell undifferentiated subtype was also related with poorer outcomes [5].

Other features may also affect prognosis in patients with HB [54], like the worse outcomes observed in C2 *vs* C1 subtype [110]. Higher CD44 expression has also been proposed as a progression marker [111]. Furthermore, worse survival rates were reported in patients with β-catenin that has lost its function as a cell adhesion molecule and accumulates in the cytoplasm [112, 113], a mutated/up-regulated *NFE2L2* [8, 54, 110], hypermethylation of *MT1G* promoter [114] or highly methylated-CpG sites of *NDRG2* and its consequent suppression [115]. The presence of membranous EpCAM with nuclear β-catenin and younger diagnostic age of HB are predictive of better response to chemotherapy as serum AFP levels drop after treatment [112]. On another hand, low *LIN28B* and miRNA let-7 expression, and high HNF1α activity have been associated to low-risk tumors with better prognosis [54].

2.2. DRUG RESISTANCE IN HEPATOBLASTOMA

2.2.1. Mechanisms of chemoresistance

Refractoriness of HB to antitumor chemotherapy is a serious problem when treating oncologic patients. The lack of response can be due to presence in the tumor of the multidrug resistance (MDR) phenotype, conferred by complex and powerful defense mechanisms, called mechanisms of chemoresistance (MOCs), which can be intrinsically present in tumor cells or can be developed by tumor cells to survive exposure to toxic compounds, *i.e.*, antitumor drugs [116]. Changes in the expression levels or the presence of genetic variants of genes involved in MOCs can markedly impact on the treatment outcome [91, 117].

To better understand MOCs, our group has classified them into seven groups that include: i) a reduction in the intracellular content of the drug and, therefore, of its ability to reach its intracellular target, either due to a minimized expression and/or function of plasma membrane solute carriers (SLCs) involved in drug uptake (MOC-1a) or to an enhanced expression and/or function of transporters of the superfamily of ATP-binding cassette (ABC) proteins involved in drug export (MOC-1b); ii) a reduced active drug content in tumor cells as a result of changes in enzymes responsible its metabolism, either a decrease expression/function in the ones involved in pro-drug activation or an increase in the inactivating ones (MOC-2); iii) alterations in the expression or function of antitumor drug's molecular targets (MOC-3); iv) enhanced ability to repair DNA damage induced by the drug (MOC-4); v) imbalance of the expression or function of pro- and anti-apoptotic factors that promote cell survival (MOC-5), which can be due to a reduction of the pro-apoptotic factors (MOC-5a) or an increment of the anti-apoptotic ones (MOC-5b); vi) alteration in the microenvironment surrounding the tumor (MOC-6); vii) activation of phenotypic epithelial-mesenchymal transition (EMT) or acquisition of stem cell characteristics by tumor cells (MOC-7) [91, 116] (**Figure I5**).
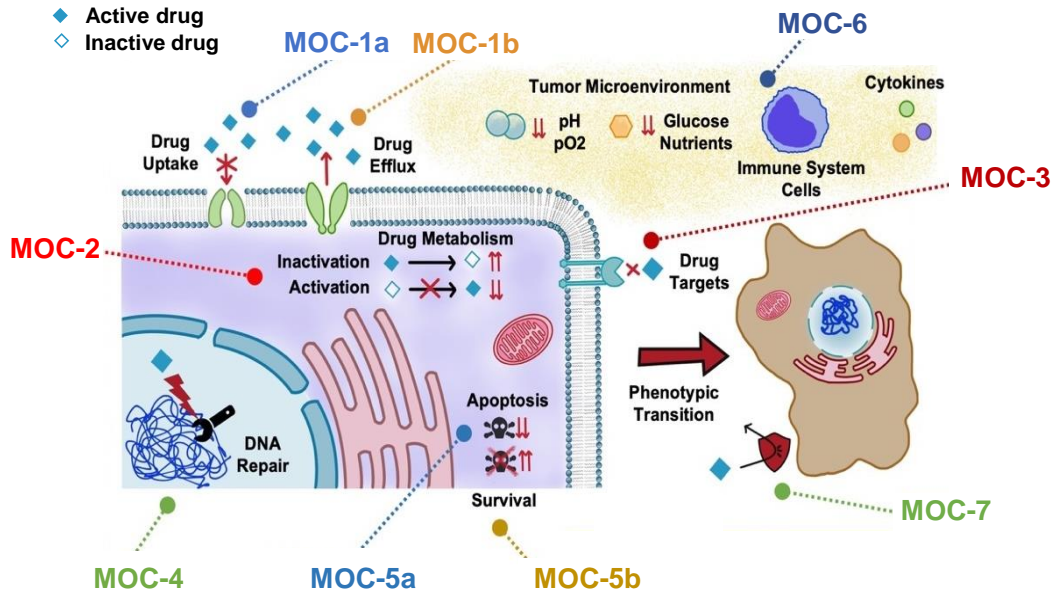
Figure I5. Schematic representation of the mechanisms of drug resistance (MOC) in cancer. Modified from [118].

MOCs responsible for the lack of response to chemotherapy are still not well known in HB. This thesis was focused on studying MOC-1.

2.2.2. Role of the transportome in chemoresistance

The "cancer transportome" can be defined as the set of transporters expressed at a given moment in the tumor. It is one of the factors underlying the MDR phenotype because impaired drug uptake and/or enhanced export can reduce intracellular drug concentration. Most drugs require transporters to enter the cell, as they are charged in aqueous solution, and, therefore, cannot diffuse freely across the plasma membrane. Consequently, the usefulness of determining the expression or function of plasma membrane transporters in cancer cells as diagnostic and prognostic biomarkers or as targets to design chemosensitizing strategies has been evaluated in several studies [119].

2.2.2.1. Carriers involved in drug uptake: the SLC superfamily of proteins

The Solute Carrier (SLC) superfamily is the second largest family of membrane proteins after G protein-coupled receptors, with over 400 members in humans, which are currently grouped into 66 families based on sequence similarities and function, ranging between 1 and 53 genes per family [120-122].

According to their transport mechanism, SLC transporters are generally categorized as facilitative transporters, but also secondary active transporters, since they use energy

generated by primary active transporters for transporting their substrates across biological membranes [123]. While secondary active transporters couple solute translocation to the cotransport or counter-transport of either inorganic ions or other solutes (exchangers), facilitative transporters translocate solutes across the membrane according to their electrochemical gradient [124]. Some SLC transporters are dependent on ion gradients, such as sodium, chloride, or protons, whereas others are solute exchangers. However, the transport stoichiometry of many of them has not been established yet. In addition, the SLC27 family also possesses enzymatic activity, whereas other members, for example those of the SLC1A family, function as ion channels, which increases the complexity of the function of this superfamily of transporters [125].

The structure of SLC proteins is characterized by transmembrane domains (TMD) (whose number varies) connected by intra and extracellular loops [119]. Unlike other superfamilies such as the ABC transporters, members of the SLCs exhibit a highly diverse structure, consisting of a wide variety of folds, many of which are evolutionarily unrelated to each other. The most common structural folds are those of the Major Facilitator Superfamily (MFS) and the Leucine transporter (LeuT). The MFS fold usually contains 12 transmembrane helices and is one of the most common membrane protein folds in nature. On the other hand, the LeuT-like fold possesses 10 TMD, with two inverted pseudorepeats of five TMD [126].

SLCs are expressed in many tissues throughout the body, although they are particularly essential in the epithelial cells of the kidney, liver, intestine, and brain. Their role in biological barrier cells is crucial in maintaining body homeostasis [123]. They are able to transport a wide variety of substrates including both endogenous compounds, such as nutrients, metabolites (neurotransmitters, vitamins, cofactors, etc.), ions, and metals ions like copper, iron, manganese, and zinc, and xenobiotics [122, 124]. Many SLC transporter families overlap in terms of solute specificity, primarily those responsible for amino acid uptake. Other members of this superfamily transport ions across the plasma membrane and even solutes across the membranes of cell organelles. Nearly half of the SLC protein families are involved in drug detoxification and therefore have a major influence on drug disposition and thus on their clinical efficacy [125, 127]. Many of these transporters are members of the SLC21/SLCO gene family (organic anion-transporting polypeptides, OATPs), the SLC22A gene family (organic anion transporters, OATs; organic cation transporters, OCTs; and organic cation/carnitine transporters, OCTNs), and the SLC15A gene family (peptide transporters, PEPTs). The SLC47A gene family (multidrug and toxin extrusion, MATEs) mediates the efflux of organic cations [123].

Although there are many different transporters involved in the uptake of antitumor agents, in this Doctoral thesis we will focus on those that have an important expression in the liver and could transport drugs used in the treatment of this cancer, such as, certain OATPs, OCTs and a copper transporter of the SLC31 family [91] (**Table I2**) (**Figure I6**).
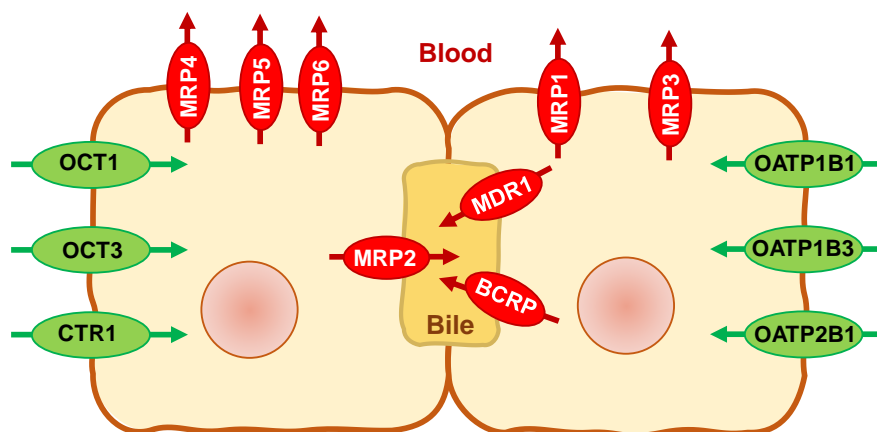
Figure I6. Schematic representation of the cellular localization of drug uptake (green) and efflux (red) transporters of interest in hepatocytes, either in the basolateral membrane (dark brown) facing the blood (sinusoidal), or in the apical membrane (light brown) in contact with bile [128-130].

OATPs

Up to date, 11 human OATPs, encoded by *SLC21/SLCO* genes [131], have been identified and divided into six families (OATP1-OATP6) based on phylogenetic relationships (more than 40% of amino acid sequence identity) and chronology of identification. Each family can have several subfamilies (more than 60% of amino acid sequence identity, such as OATP1A, 1B, and 1C) [123].

The structure of OATPs is characterized by a 12-TMD that includes the superfamily signature, a large extracellular loop between TMD9 and 10 with 11 conserved disulfide-bonded cysteine residues, and multiple N-glycosylation sites in the extracellular loops 2 and 5 [132, 133]. Their superfamily signature, located at the extracellular border of TMD6, consists of 13 well conserved amino acids: D-XRW-(I,V)-GAWWX-G-(F,L)-L [132] (**Figure I7a**).

In general, OATP substrates are anionic amphipathic molecules with a rather high molecular weight (>350 Da) and a high degree of albumin binding under physiological conditions, as these transporters contain two hydrogen bond acceptors, one hydrogen bond donor, and two hydrophobic regions [131]. OATPs are exchangers, as they couple

the uptake of substrates to the efflux of another anion such as HCO_3^- or reduced glutathione (GSH) [134, 135].

Although the exact common transport mechanism of OATPs has not been established yet, it has been suggested to be of a rocker-switch type through a positively charged central pore. In this mechanism, two major alternating conformations are considered in the transporter: inward-facing and outward-facing, conversion between the two would only be possible via a substrate-bound protein form. The single binding site in a pore is only accessible from one side of the membrane [132]. OATP-mediated organic anion exchange seems to be pH-dependent and electroneutral [123, 136].
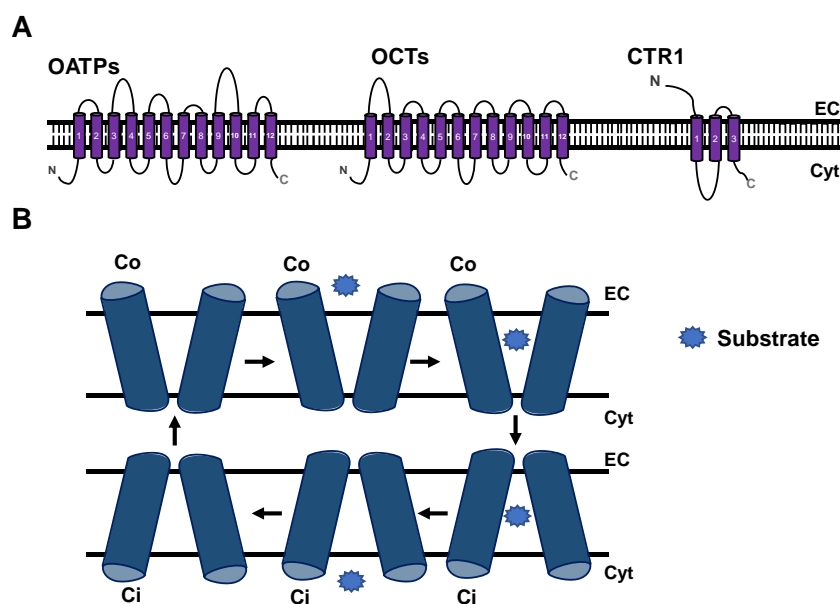
Figure I7. (**A**) Secondary structure models of transporters members of the organic anion transporting polypeptide (OATP) family, organic cation transporter (OCT) subfamily and copper transporter 1 (CTR1). Transmembrane domains (TMD) are in purple. (**B**) Schematic representation of the conformational changes that OCT transporters undergo in the presence of a substrate (Co, outward-facing conformation and Ci, inward-facing one). C, carboxy-terminus; Cyt, cytoplasm; EC, extracellular compartment; N, amino-terminus.

In the liver, **OATP1B1** (formerly called OATP-C), encoded by the *SLCO1B1* gene, and **OATP1B3** (formerly called OATP8), encoded by the *SLCO1B3* gene, are responsible for hepatic uptake from the blood of many endogenous compounds like bile acids, conjugated and unconjugated bilirubin, thyroid hormones, eicosanoids, and steroids, in addition to anionic drugs [123, 137]. Some of the most important therapeutic drugs that are substrates of OATPs include HMG-CoA inhibitors such as statins [138]. These two transporters are almost exclusively expressed on the sinusoidal (basolateral) membrane of hepatocytes. In fact, OATP1B1 and OATP1B3 were previously named as liver specific transporter 1 (LST-1) and 2 (LST-2), respectively. Interestingly, the expression of a

splicing variant of OATP1B3 called "Cancer-Type" was also found in some tumors including gastric, colon, and pancreatic cancers [139]. Regarding antitumor agents used to treat HB that may be substrates of these transporters, some studies have shown that OATP1B1 and OATP1B3 are involved in the cellular uptake of platinum derivatives, such as cisplatin, carboplatin and oxaliplatin [140]. Cisplatin in aqueous solution forms mostly positively charged compounds. However, it is possible that other neutral or negatively charged derivatives are also formed, which would be potential substrates for OATPs. There is controversy in this regard, as other studies claim that OATPs lack the ability to take up platinum compounds [140-142].

In vitro studies in human hepatocytes have shown that a small part of vincristine uptake by these cells is mediated by OATPs (**Table I2**) [143]. OATP1B1 and OATP1B3 are involved in the uptake of some TKIs, like sorafenib and regorafenib [144]. Therefore, decreased expression of OATP1B1 and OATP1B3 may play a role in the resistance of HB to drugs transported by these proteins.

OATP2B1, encoded by the *SLCO2B1* gene, is a ubiquitously expressed uptake transporter with broad substrate specificity. Its high expression in the sinusoidal membrane of hepatocytes and the apical membrane of enterocytes suggests a relevant role in drug pharmacokinetics and toxicology, although these aspects are still not well understood [145]. Its transport mechanism is strongly influenced by extracellular pH, as it seems to transport some substrates more efficiently at lower pH. Its substrate specificity appears somewhat more restricted than those of OATP1B1 and OATP1B3 and partly overlaps with that of these transporters [146]. Although statins are substrates of OATP2B1, its ability to transport antitumor drugs is not well known [147]. It seems to play a minor role in chemoresistance. This transporter could be involved in the uptake of vincristine [143] and irinotecan, as inhibition of OATP2B1-mediated transport protects against drug-induced toxicity in intestinal tissue [148].

OCTs

OCT1-3 proteins, encoded by *SLC22A1-3*, are members of one of the subtypes of the SLC22 family. OCT1 and OCT2 amino acid sequences have 70% identity and around 50% identity with OCT3 [123, 135].

They contain 12 TMDs, a large extracellular loop between TMD1 and TMD2 (with glycosylation sites) that mediates homo-oligomerization, and a large intracellular loop between TMD6 and TMD7 (with phosphorylation sites) [135, 149]. N-glycosylation may be involved in protein stability, intracellular trafficking, or protection from extracellular

proteases [150]. Amino and carboxy termini of the protein are both intracellular [123] (**Figure I7a**).

OCT1-3 are facilitative diffusion systems that translocate organic cations down their electrochemical gradients in both directions across the plasma membrane. The "simple alternating access" mechanism of OCT-mediated transport involves inward and outward conformations with binding pockets that contain overlapping interaction domains for organic cations [151] (**Figure I7b**). OCT transport is independent of either sodium gradients or pH. They always behave as electrogenic carriers for charged substrates [135].

OCT1 and **OCT3** are both localized in the basolateral membrane of hepatocytes [123]. Their substrates include a wide variety of structurally unrelated small organic cations, both endogenous and exogenous compounds, including many drugs [135]. There is some overlap between OCT1 and OCT3 substrate-specificity. While OCT1 substrates are organic cations (mainly monovalent), such as tetraethylammonium (TEA) [152], some weak bases and non-charged compounds [149], OCT3 transports mainly endogenous compounds and neurotransmitters [130]. Regarding antitumor drugs, OCTs are involved in doxorubicin and cisplatin transport [123, 153, 154]. OCT2 is primarily expressed in the kidney, where it is accounted for cisplatin toxicity [155]. OCT1 is also responsible for sorafenib [156] and irinotecan [157] transport, while vincristine might interact with OCT3 [158].

In HepG2 cells, OCT3 expression levels correlate with intracellular accumulation of cisplatin and the sensitivity to this drug, suggesting that OCT3 may play a role in the response to cisplatin [159]. As in other liver tumors, a marked decrease in OCT1 expression has been described in HB compared to expression in adjacent non-tumor liver tissue [91, 160]. Decreased OCT1 expression has been associated with decreased sensitivity to drugs taken up by these transporters in different tumors, although its relevance in HB has not yet been investigated.

CTR1

Copper transporter 1 (**CTR1**), encoded by *SLC31A1* gene, is a major transporter accounting for cisplatin uptake [123, 161]. Under physiological conditions, CTR1 was characterized by its high affinity for reduced copper, being responsible for the cellular uptake of at least 80% of this metal.

This transporter has a long extracellular N-terminus that contains N- and O-glycosylation sites as well as two methionine-rich and two histidine clusters (both potential metal-

binding residues), three transmembrane segments, and a cytosolic C-terminal tail [128, 162] (**Figure I7a**). CTR1 monomers assemble into trimers to form a structure containing a pore for metals to cross the membrane [162, 163]. This multimeric complex changes its conformation to transport substrates: the axes around the pore center rotate and the cytoplasmic part of the helixes move away from the pore [128]. It is known to be broadly expressed, with high levels in the liver, located in the basolateral membrane of hepatocytes [128, 162].

Loss of CTR1 expression has been shown to cause the development of cisplatin resistance [164].

Table I2. Major drug uptake transporters of the SLC superfamily expressed in hepatocytes capable of transporting antitumor drugs used in patients with hepatoblastoma.

Pharmacological group	Drugs	Uptake transporters	References
Anthracyclines	Doxorubicin	OCT1, OCT3, OCTN1, OATP1A2	[153, 165]
	Epirubicin	N.D.	
Platinum derivatives	Cisplatin	OCT1, OCT3, CTR1	[144]
	Carboplatin	CTR1	[166]
Alkylating drugs	Cyclophosphamide	N.D.	
	Ifosfamide	N.D.	
Camptothecins	Irinotecan	OAT2	[165]
Tyrosine kinase inhibitors	Cabozantinib	N.D.	
	Lenvatinib	N.D.	
	Regorafenib	OATP1B1	[144]
	Sorafenib	OCT1, OATP1B1, OATP1B3	[144, 156]
Podophyllotoxins	Etoposide	OCTN2	[165]
Pyrimidine analogs	5-Fluorouracil	ENT1, ENT2, OAT2	[165, 167]
Vinca alkaloids	Vinblastine	N.D.	
	Vincristine	OCT3, OATP1B1, OATP1B3	[143]

N.D., Not determined.

2.2.2.2. Proteins involved in drug efflux: the ABC superfamily

ABC proteins are primary active transporters driven by the energy generated during ATP hydrolysis to export solutes across cell membranes without the existence of a substrate concentration gradient on the different sides of the membrane [168, 169]. The 48 proteins of the ABC superfamily are distributed in seven families of ABC proteins (from A to G) in humans [124] based on their catalytic site structure and sequence similarities [170].

The structure of ABC proteins is characterized by two domains: TMDs, consisting of several highly hydrophobic alpha-helices that recognize and transport the substrates, and bulky nucleotide-binding domains (NBDs), in which ATP molecules bind and are

hydrolyzed. Two subdomains can be distinguished within NBD: i) a catalytic core domain for the binding and hydrolysis of ATP with conserved motifs called Walker A (rich in glycine), Walker B (characterized by 4 hydrophobic residues followed by a glutamate) and H as well as A, Q, D, loops and ii) an α-helical domain that contains the ABC signature motif or C-loop (LSGGQ) involved in binding the nucleotide (**Figure I8a**). NBDs, which are located facing the cytoplasm, are highly conserved in all families (and between organisms), but the TMDs are very heterogeneous, which confers versatility to the pumps [129, 171-174].

ABC transporters are organized as full transporters with two domains of each type (alternating TMD and NBD) or as half transporters with one (TMD-NBD) that must form either homo- or heterodimers to function (**Figure I8b**) [129]. The extracellular side of ABC transporters is, to a greater or lesser extent, N-glycosylated, but this does not seem to be necessary for transport but for folding and stabilization of the protein during its translocation to the plasma membrane [129, 175].

Their export mechanism is not completely understood, but the "ATP switch" model is the most accepted as it explains the functional "conformational changes" of ABC transporters [129]. This model is based on the binding of the substrate to the TMD and an ATP molecule to each NBD, where it is hydrolyzed to ADP and phosphate to produce a translocation that allows the export of the substrate from the cell interior. In the end, the initial structure is restored [176] (**Figure I8c**).

ABC proteins are widely expressed in human tissues such as the liver, intestine, kidney, and brain and within the cell, primarily located on the plasma membrane, although some are found in the membrane of organelles (mitochondria, lysosome, peroxisome, or endoplasmic reticulum) [129].

A

B

C
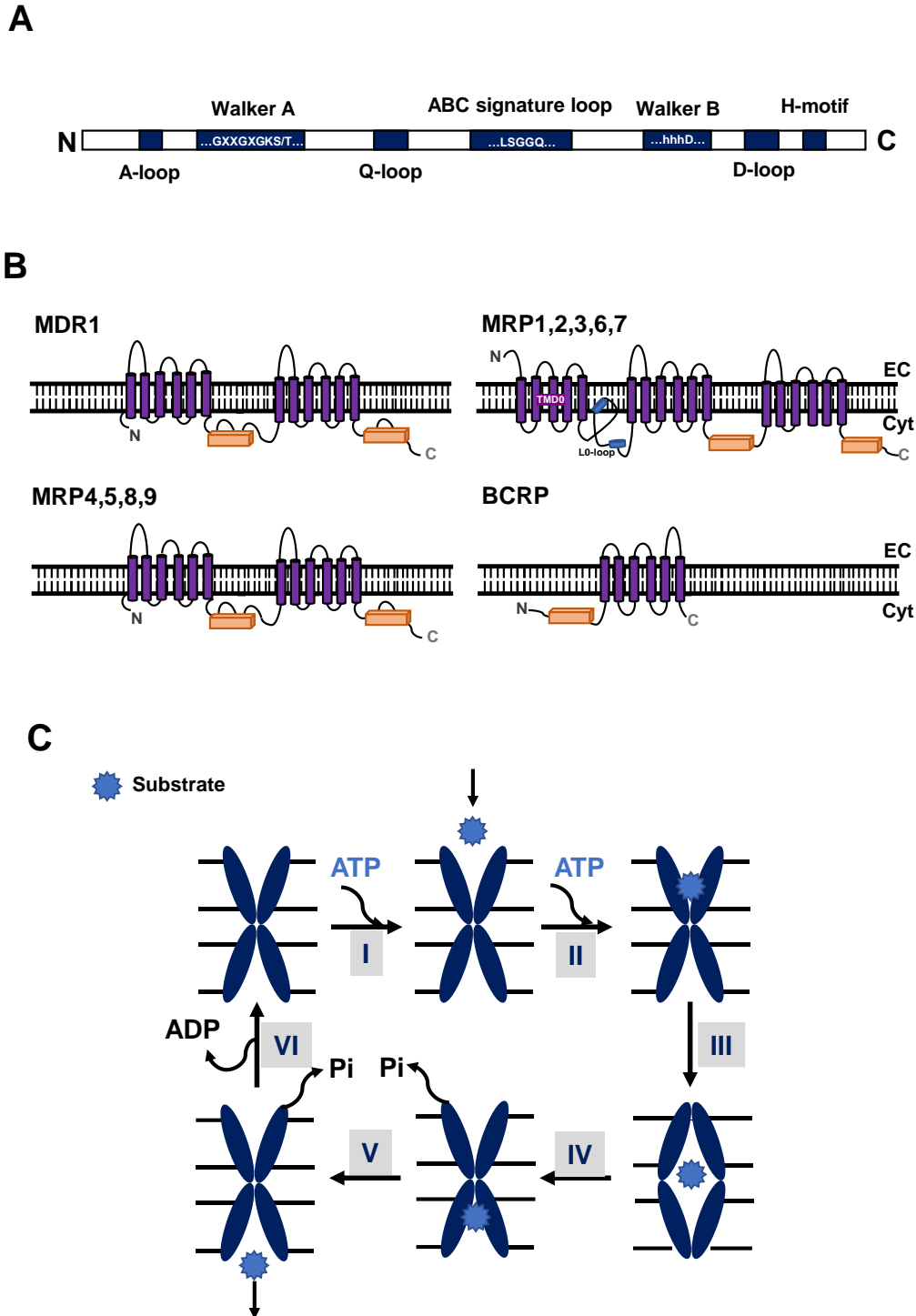
Figure I8. (**A**) Primary structure of nucleotide-binding domain (NBD) with characteristic residues within its elements. (**B**) Secondary structure models of drug efflux transporters members of the ATP-binding cassette family. Transmembrane domains (TMD) in purple, NBD in orange. (**C**) ATP switch model to explain ABC pumps mechanism of ATP binding and hydrolysis by NBD dimers: (I) resting state; (II) the monomers are separated in the absence of ATP; (III) the monomers are loaded with ATP, the dimers form a sandwich to close the interface; (IV) processive hydrolysis of ATP in each site; (V) sequential release of Pi and ADP; (VI) return to the nucleotide-free open state. The substrate is represented in light blue. C, C-terminus; Cyt, cytoplasm; EC, extracellular compartment; h, any hydrophobic residue; N, N-terminus.

Among their substrates, there are endogenous compounds (inorganic ions, polysaccharides, lipids, amino acids, peptides, nucleosides, and steroids) [172], as well as exogenous substances such as drugs and toxins [177]. In fact, some ABC pumps are part of the carriers accounting for reduced drug and xenobiotic disposition to protect tissues. Moreover, ABC proteins play a crucial role in maintaining cell homeostasis, cholesterol trafficking, immunity, and cell division [129].

Some members of ABCB, ABCC or ABCG families are involved in drug export and, hence, in cancer chemoresistance [119, 127, 129, 178]. The main pumps known to be involved in reducing the intracellular content of anti-tumor agents in liver cancer are multidrug resistant protein 1 (MDR1, *ABCB1*), multidrug resistant-associated proteins 1 to 6 (MRP1-6, *ABCC1-6*) and breast cancer resistant protein (BCRP, *ABCG2*) [91, 179] (**Table I3**).

MDR1

MDR1, also known as P-glycoprotein or P-gp, encoded by *ABCB1*, was the first human ABC transporter identified because of its ability to confer chemoresistance in cancer cells [180]. It contains 1280 amino acids (170 kDa) [181]. *ABCB1* is highly polymorphic, being these single nucleotide polymorphisms' (SNP) allele frequency different among ethnic groups. The protein has two NBDs and TMDs and a heavily N-glycosylated first extracellular loop. MDR1 restricts the entry of its substrates into organs such as the brain, testis, placenta, and gastrointestinal tract. In addition, it removes substrates from organs with a major detoxifying function, mediating both biliary and renal excretion [129]. This transporter has a very wide spectrum of substrates, preferentially neutral or positively charged hydrophobic molecules, mediating the export of a variety of drugs including anti-tumor ones used to treat HB, like doxorubicin, *Vinca* alkaloids (vincristine, vinblastine), cyclophosphamide, ifosfamide, irinotecan, and etoposide [175, 182-184]. However, MDR1 is not believed to be a cisplatin transporter. Some TKIs used to treat liver cancer in adults, like cabozantinib [184], sorafenib [185], regorafenib [186] and lenvatinib [187] are drugs that interact with MDR1 and decrease or block its activity [188] and could even be transported as well.

Some studies with recurrent HB tumor samples have found an up-regulation of MDR1 after exposure to standard chemotherapy [189]. *ABCB1* up-regulation has also been observed after treatment of HB xenograft-bearing mice with cisplatin and doxorubicin [190]. Results obtained in an experimental model of MDR1 overexpression in HepG2 cells showed the relevance of this ABC protein in doxorubicin resistance [182].

MRPs

MRP proteins are members of the largest ABC family, ABCC. The MRP family consists of 13 members, of which MRP1 to MRP9 are transporters that may contribute to the MDR phenotype in tumor cells. The best known are MRP1 to MRP6, which are encoded by *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC5*, *ABCC6*, respectively. MRP4 and MRP5 have a typical ABC structure (TMD1-NBD1-TMD2-NMD2) and are usually N-glycosylated on the fourth extracellular loop. However, MRP1, -2, -3, -6 and -7 have an extra domain (TMD0) composed of five transmembrane helices, linked to TMD1 by a L0-loop, and their extracellular N-terminus is also glycosylated [129, 191, 192]. They are transporters of mainly lipophilic anionic compounds, often conjugated with GSH, glucuronate or sulfate. In addition, MRP1 and MRP2 can transport drugs with no net electrical charge in the presence of GSH. MRPs can transport structurally unrelated drugs, including naturally occurring drugs, nucleoside analogs, antimetabolites, and TKIs [178].

MRP1 was originally identified as the mediator of doxorubicin resistance in a small-cell lung cancer cell line. MRP1 contains 1531 amino acids (190 kDa) and is both N-glycosylated and phosphorylated. MRP1 is ubiquitously expressed in humans, with testis, placenta, prostate, lung, and kidney being the organs where the highest mRNA levels have been found. Its expression in healthy liver is low. MRP1 plays an important role in the development of drug resistance in several types of cancer [193]. MRP1 transports structurally diverse amphipathic organic anions, most of which are conjugated with GSH, glucuronide, or sulfate, carrying out phase III drug elimination as these conjugates are usually products of phase II drug metabolism. Free GSH and glutathione disulfide (GSSG) transport is also mediated by this pump [129]. MRP1 activity has been related to resistance to anthracyclines (doxorubicin, epirubicin, idarubicin), *Vinca* alkaloids (vincristine and vinblastine), etoposide, 5-FU, and irinotecan [194-197]. There is no direct evidence on the ability of MRP1 to transport TKIs, however, some of these drugs can inhibit its activity [192].

MRP1 expression has been shown to be an important factor in anthracycline resistance in different types of cancer [197]. However, its relevance in HB chemoresistance is poorly understood. An association has been found between MRP1 overexpression and decreased sensitivity to doxorubicin in HB-derived HuH6 cells [198].

MRP2, cMOAT or cMRP (canalicular multispecific organic anion transporter) is a 1545-amino acid (190 kDa) protein [181] containing a lysine-rich element essential for apical targeting in the cytoplasmic region that links TMD0 to TMD1 [199]. It is located at the

apical membrane of polarized epithelial and endothelial cells, so it is primarily expressed at canalicular membrane of hepatocytes [200, 201]. Its expression is also detected in some tumors, such as renal, hepatocellular, ovarian, and colorectal carcinomas [202]. MRP2 substrate specificity is very similar to that of MRP1 [129]. MRP2 substrates include anticancer drugs like anthracyclines, irinotecan, some TKIs (sorafenib, lenvatinib, regorafenib, cabozantinib), etoposide, and *Vinca* alkaloids [91, 179, 203-206] -– most of them are conjugated with glucuronate, sulfate, or GSH [129]. Notably, unlike MRP1, MRP2 can mediate the efflux of glutathione-conjugated cisplatin, and overexpression of MRP2 has been associated with cisplatin resistance in cancer cell lines [207].

MRP3 consists of 1527 amino acids (190-200 kDa). It is expressed in basolateral membranes of polarized cells in different tissues, including hepatocytes and enterocytes, where it participates in the transport of quite a broad range of organic anions both endogenous (like bile salts) and xenobiotic (drugs like etoposide and some TKIs). Interestingly, MRP3 does not require GSH for their substrates to be transported [129].

MRP4 is the smallest of the MRP proteins with 1325 amino acids (170 KDa) [208, 209]. It is widely expressed in most tissues. In the liver, it localizes at the basolateral membrane of hepatocytes. This pump can transport many drugs as well as endogenous molecules like cyclic nucleotides, ADP, and eicosanoids, which affect cell proliferation, differentiation, and apoptosis [129, 210]. MRP4 could be involved in resistance to 5-FU and irinotecan, and perhaps also to doxorubicin and cyclophosphamide [91]. Although cisplatin is not a known substrate of MRP4, it was upregulated in a cisplatin-resistant cells from HCC, so it can be involved in developing cross-resistance [179].

MRP5 contains 1437 amino acids (185-200 kDa) [211]. It is expressed at low levels in most normal tissues including the liver. It transports cyclic nucleotides (with a higher affinity for cGMP than cAMP) and nucleotide analogues [129] leading to resistance to drugs derived from pyrimidine bases, like 5-FU [212]. Anthracyclines could also be MRP5 substrates [208].

MRP6, a 190 kDa protein, is expressed in the basolateral membrane of hepatocytes. Its importance in conferring drug resistance is yet to be determined. Cells over-expressing this transporter were slightly less sensitive to cisplatin, anthracyclines and etoposide [129, 213, 214].

BCRP

BCRP or MXR (mitoxantrone resistance-associated protein), encoded by *ABCG2*, was first identified as a transporter that conferred resistance to doxorubicin in breast cancer

cells [215]. BCRP is a half transporter of 655 amino acids (70 kDa) that forms homodimers to function and is N-glycosylated in the last extracellular loop. Its tissue distribution markedly overlaps with that of MDR1; in the liver, it is highly expressed in the canalicular membrane of the hepatocytes [129, 216]. BCRP substrates include a broad spectrum of anticancer drugs (such as anthracyclines, irinotecan, etoposide, sorafenib, and 5-FU), sulfate and glucuronide conjugates of sterols and xenobiotics, natural compounds, and toxins [91, 129, 185].

ATP7

Other drug efflux transporters include copper efflux transporters, encoded by *ATP7A* and *ATP7B*, that are P-type ATPases involved in platinum-derived drug resistance [217]. In addition, overexpression of *ATP7A* and *ATP7B* has been found in multiple types of cancer, mainly of the gastrointestinal tract.

Table I3. Major drug efflux transporters expressed in hepatocytes capable of transporting antitumor drugs used in patients with hepatoblastoma.

Pharmacological group	Drugs	Efflux transporters	References
Anthracyclines	Doxorubicin	MDR1, MRP1, MRP2, MRP6, BCRP	[184]
	Epirubicin	MDR1, MRP1, MRP2	[144, 203]
Platinum derivatives	Cisplatin	MRP2, MRP6, ATP7A, ATP7B, MATE1	[184, 217] [144]
	Carboplatin	MRP2, BCRP, ATP7A, ATP7B	[217, 218]
Alkylating drugs	Cyclophosphamide	MDR1, MRP1, MRP4, BCRP	[183]
	Ifosfamide	MDR1, MRP1, MRP4, BCRP	[183]
Camptothecins	Irinotecan	MDR1, MRP1, MRP2, MRP4, MRP6, BCRP	[184]
Tyrosine kinase inhibitors	Cabozantinib	MDR1, MRP2	[184]
	Lenvatinib	MDR1, MRP2	[144, 184]
	Regorafenib	MDR1, MRP2, BCRP	[144]
	Sorafenib	MDR1, MRP1, MRP2, MRP3, BCRP	[144, 184]
Podophyllotoxins	Etoposide	MDR1, MRP1, MRP2, MRP3	[184, 219]
Pyrimidine analogs	5-Fluorouracil	MRP1, MRP4, MRP5, BCRP	[184]
Vinca alkaloids	Vinblastine	MDR1, MRP1, MRP2	[184]
	Vincristine	MRP1, MRP2	[219]

ATP7, ATPase copper transporting; BCRP, breast cancer resistance protein; HB, hepatoblastoma; MATE, multidrug and toxic compound extrusion; MDR, multidrug resistance protein; MRP, multidrug resistant-associated protein; TKI, tyrosine kinase inhibitor.

2.3. CHEMOSENSITIZATION STRATEGIES

As previously mentioned, approximately 20% of patients with high-risk HB do not respond to pharmacological treatment and their survival is usually less than 3 years [1, 66, 220]. Hence there is an urgent need to find novel and more effective therapeutic alternatives, using better tumor-selective approaches.

2.3.1. Usefulness of uptake transporters as targets

Antitumor drug-substrate conjugates

Antitumor drugs can be chemically modified to facilitate their uptake into cancer cells, increasing their intracellular accumulation [221, 222]. For example, the prodrug strategy is based on conjugating a known substrate of an uptake transporter to the drug of interest to promote the entry into cells, where the active agent is subsequently released by enzymatic action [223] (**Figure I9**). On other occasions, the conjugate of the antineoplastic drug and the shuttle part of the molecule maintains the antitumor activity and it is not required to be separated [224].
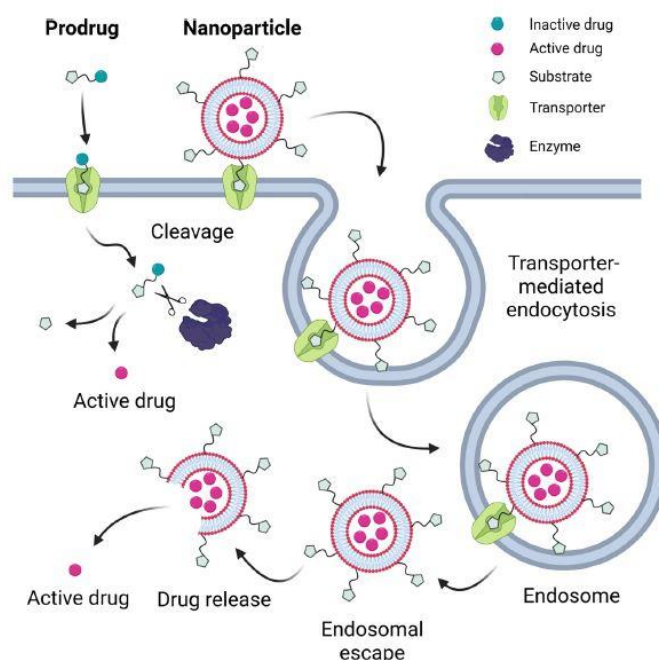
Figure I9. Schematic representation of chemosensitizing strategies: based on linking the drug of interest to a known substrate of the target transporter (a prodrug) and functionalized nanoparticles that encapsulate the active drug molecules. Obtained from [124].

OATP1B1 and OATP1B3 have been studied as targets to enhance liver-specific drug delivery [124, 225], as it could also minimize off-target side effects. Bile acids are among the substrates of these OATP transporters [226], and several studies have proposed that

drug conjugation with these endogenous compounds could help to vectorize them towards the hepatobiliary system [124, 227], since their expression is often maintained in liver tumors [228]. One example is the Bamet family of drugs that our research group has developed and characterized over the last 25 years [229-232]. They are conjugates of one or more bile acids plus a metal coordination complex, which stands for their name Bamet (BA, from "bile acid" and MET, from "metal"). Bamet-UD2 is one of those compounds which has shown to have the best antitumor properties. Bamet-UD2 was obtained by conjugating cisplatin with two ursodeoxycholate moieties. Bamets can be taken up by bile acid transporters and have a strong cytostatic activity in several tumor cell lines, including the HB-derived cell line HepG2, and in tumors implanted in mice with lower side effects than the parent drug cisplatin [119, 224, 233]. Our group also synthesized and characterized a group of compounds containing a nitrogenous base and a bile acid, named BANBs, which also demonstrated cytostatic activity and vectoriality against enterohepatic tumors [234].

Cytarabine has a poor oral absorption due to its rapid deamination and poor permeability across the plasma membrane of the epithelial cells of the gastrointestinal tract and it has also been conjugated with different bile acids to improve oral absorption. These prodrugs showed potent antiproliferative activities against HepG2 cells [235].

L-type amino acid transporter-1 (LAT1, *SLC7A5*) is involved in the transport of neutral amino acids and is highly expressed in many types of cancer. Interestingly, when aspartate was attached to the N-terminal of doxorubicin (Asp-dox), it was taken up by HB cell lines and achieved a stronger tumor growth inhibition *in vivo* that the parent drug [236].

ASCT2, a sodium-dependent neutral amino acid exchanger encoded by *SLC1A5*, is overexpressed in several cancers [237, 238]. Glutamine is a substrate of this transporter and an important metabolite for cancer cells [239, 240]. When cisplatin was conjugated with glutamine, these derivatives showed less anticancer activity than cisplatin, but also reduced off-target accumulation and side effects [124, 241].

Another promising strategy for drug delivery is based on encapsulating drugs in nanoparticles, which can release them under certain conditions, in order to increase their solubility and/or to protect them from digestive degradation.

The nanoparticles generally are taken up by the cell through binding to the target transporter or receptor on the cell surface and consequently triggering different endocytosis pathways [124, 242, 243]. It is worthy of note that the efficiency of these

endocytosed particles can be reduced if they are trapped in lysosomes, which could lead to their degradation [124, 244]. There are physiological limitations to the size of the nanoparticles: particles below 10 nm are filtered in the renal glomerulus, particles with up to 25 nm diameter can undergo endocytosis [245] and particles above 200 nm can accumulate in the liver and spleen as they activate the complement system (**Figure I9**) [246]. They are usually smaller than one micrometer [124].

Chemicals used as nanocarriers for transporter-targeted drug delivery include formulations based on liposomes or solid-lipids, various polymers, carbon dots, mesoporous silica, and nanoemulsions [124].

Bamet-R2- or Bamet-UD2-loaded liposomes proved to be useful pharmacological tools *in vitro* and *in vivo* for circumventing chemotherapy resistance in tumors of the enterohepatic circuit [247, 248].

Nanoparticle scaffolds can be chemically modified to improve drug absorption and target drugs to specific tissues, such as by binding small molecule substrates of transporters to their surface [124]. For example, poly(lactic-co-glycolic acid) based-nanoparticles modified with human serum albumin can increase the cellular uptake by receptor-mediated endocytosis [222, 249].

Nanoparticles have been conjugated with different amino acids. Phenylalanine is a LAT1 substrate, so this amino acid was conjugated to solid lipid nanoparticles intended to encapsulate doxorubicin to treat glioma [250, 251]. Lysine-conjugated liposomes with docetaxel, which seem to interact with $ATB^{0,+}$ (encoded by *SLC6A14)* and be later introduced in the cell by endocytosis, showed to be taken up by HepG2 cells and had greater efficacy and less systemic toxicity [243]. To target both LAT1- and $ATB^{0,+}$-mediated endocytosis, functionalized liposomes conjugated with different amino acids were loaded with irinotecan, achieving highest uptake efficiency with tyrosine-functionalized ones in breast cancer cells [124, 252]. LAT1 and LAT2 can transport methionine, so gold nanoclusters were modified with this amino acid and charged with doxorubicin; both anti-tumor activity and tumor affinity were enhanced *in vitro* in a liver cancer derived cell line [253, 254].

Vitamin uptake is increased in several types of cancer cells. Biotin-conjugated doxorubicin nanoparticles made of pullulan acetate, a hydrophobized polysaccharide, increased uptake in HepG2 cells [255]. Biotin-coated carbon-based nanomaterials ("nanodiamonds") were also developed and could be a good vehicle for antitumor drugs [124, 256].

Doxorubicin-loaded micelles with dehydroascorbic-1 acid, a glucose transporter 1 (GLUT1) substrate, achieved enhanced anti-tumor activity in HepG2 cells and *in vivo* [257]. Glucose-coated magnetic nanoparticles were also developed [258].

Pirarubicin conjugated with hydroxypropyl acrylamide polymer selectively releases active drug molecules into an acidic environment (typically surrounding the tumor), which can reduce toxicity and improve efficacy in pediatric tumors, such as HB [259].

Etoposide was embedded in a nanoemulsion obtained with methylcellulose, 1,2-didecanoyl-sn-glycero-3-phosphate and N^{α}-deoxycholyl-L-lysylmethylester. This resulted in an enhanced ability to cross the intestinal barrier. Accordingly it is expected to have more oral bioavailability than the commercially available etoposide emulsion [260].

Changing transporter expression

As the downregulation of some SLC transporters can affect drug efficacy, novel gene therapy approaches have been developed to enhance the expression of the transporter of interest. This was the case of OCT1, whose expression is low in liver tumors, which limits the response of HCC to drugs taken up by this transporter, such as sorafenib [160, 261]. In experiments carried out in our group to manipulate OCT1 expression, lentiviral vectors carrying OCT1 coding sequence were used to transduce HepG2 cells [262], while adenoviral vectors bearing the OCT1 coding sequence under the transcriptional control of the tumor-specific *BIRC5* promoter were constructed and essayed *in vivo*, with cholangiocarcinoma-bearing mice [263]. Both approaches resulted in an increased OCT1 expression and consequently an enhanced sorafenib uptake and response [262, 263].

Altering nutrient and metabolite uptake by tumor cells

In another line of investigation, some transporters have been targeted to alter cancer cell metabolism and, hence, inhibit tumor growth [119].

The so-called Warburg effect states that cancer cells preferentially undergo glycolysis. Thus, transporters involved in glucose uptake could be targeted. Berberine, a component of numerous traditional Chinese medicinal herbs, markedly decreased the glucose uptake ability (through GLUT1), reduced ATP synthesis, and effectively inhibited proliferation of HepG2 cells [264].

Solute carrier family 13 member 5 (SLC13A5) is an uptake transporter mainly expressed in hepatocytes that takes up citrate from blood circulation, which plays a role in

metabolism, cell proliferation, and stress response. HB-derived cells with silenced SLC13A5 were more sensitive to cellular stress caused by cisplatin, doxorubicin, 5-FU and sorafenib [265].

2.3.2. Usefulness of efflux transporters as targets

ABC-pumps' inhibitors

The co-administration of antitumor drugs and ABC pumps' inhibitors could increase the intracellular content of the drug and thus its cytostatic activity, which could help to overcome chemoresistance caused by high activity of these transporters in tumor cells (MOC-1b). Therefore, these chemosensitizers have been investigated and developed with this aim since the role of drug efflux pumps in tumor chemoresistance was discovered [119, 222, 266]. However, although some ABC-inhibitors have been tested in clinical trials, none of them have been approved to treat any chemoresistant tumor yet [222].

There is more information regarding MDR1 inhibitors than other ABC efflux pumps' modulators. Some inhibitors are also substrates and compete with drug-binding sites, whereas others are poorly transported and act through different inhibition mechanisms [129, 179]. MDR1 inhibitors can be classified into three generations as they have been developed over time, improving their specificity, affinity and reducing their toxicity [222].

Already clinically approved drugs for the treatment of other pathologies that are also MDR1 substrates, like verapamil, quinidine, and cyclosporin A, were repurposed as inhibitors of this pump and constituted the first generation [267]. However, high concentrations were required to inhibit drug efflux and caused severe adverse effects due to their low specificity [119, 129, 222, 267, 268]. Both quinine and verapamil are cardiotoxic [269], while cyclosporine A is nephrotoxic and immunosuppressive [270].

These promising compounds were subsequently chemically modified to create a second generation of inhibitors to target more specifically drug efflux transporters with lower side effects. For example, valspodar (previously known as PSC-833) is a more potent derivative of cyclosporine A [271], although it failed to improve the efficacy of standard anticancer drugs [272]. Besides, its high affinity for ABC transporters like MDR1 and BCRP, valspodar also inhibits CYP3A4, a cytochrome P450 enzyme, so despite showing less toxicity than the parent drug, off-target effects were also detected [119, 222].

Consequently, a third generation of inhibitors was designed using combinatorial chemistry and subsequent structure-activity relationship studies to improve their

potency, specificity, and low toxicity [222, 266]. Those needs were met as the compounds developed efficiently inhibit ABC pump-mediated drug efflux at nanomolar concentrations and they did not inhibit CYP enzymes [273, 274]. Elacridar (GF120918), laniquidar (R101933), tariquidar (XR-9576) and zosuquidar (LY-335979) are examples of chemosensitizers belonging to this group, which were evaluated in clinical trials [268, 275].

Elacridar effectively increased doxorubicin and paclitaxel efficacy in xenograft tumors [276] as well as topotecan bioavailability in mice and breast cancer patients [276-278]. Clinical trials testing tariquidar were cancelled due to an increased toxicity and lack of efficacy [279-281] despite its inhibitory ability *in vitro* [279, 282, 283]. Zosuquidar improved the sensitivity to different antitumor drugs, like doxorubicin, etoposide, and paclitaxel *in vitro* [284], but did not achieve as good results when tested in cancer patients [285, 286].

Few MRPs' inhibitors with potential usefulness in patients have been described, as their bioavailability is low due to their poor ability to cross the plasma membrane and reach their intracellular targets. Examples include probenecid, MK-571 and diclofenac [129]. Modulation of intracellular GSH levels through MRP1 has been proposed as a sensitizing strategy in cancer therapy. Verapamil is not transported by MRP1 but is able to stimulate GSH efflux mediated by this pump. GSH extrusion triggers a selective apoptosis of cells overexpressing MRP1 [287].

Regarding BCRP inhibitors, fumitremorgin C was the first natural compound isolated with such function, although it cannot be used in patients due to its neurotoxicity. Both elacridar and tariquidar are able to inhibit BCRP, along with some flavonoids and precursors like chalcones and derivatives [288].

Unfortunately, many inhibitors that have been tested in clinical trials have failed in spite of promising preclinical data due to i) high variability in response rate; ii) toxicity of a co-administered drug in healthy tissues; iii) non-specific toxicity [129, 222].

Thus, the search for efficient and non-toxic chemosensitizers among nutraceuticals (compounds from natural sources) and synthetic compounds continues. In fact, some products of natural origin that inhibit ABC-mediated efflux of drugs include curcumin, some polyphenolic compounds and trabectedin [222, 268, 289]. For example, polyethylene glycol nanoparticles labelled with biotin carrying both curcumin and doxorubicin were designed to improve the delivery of both the chemosensitizer and the chemotherapeutic agent in breast cancer cells [290].

Moreover, myricetin reduced oxaliplatin resistance in HepG2 cells by inhibiting MRP2 [291], while capsaicin and piperine chemosensitized colon cancer cells to doxorubicin [292]. Similarly, isopetasin in leukemia cells [293] as well as saikosaponin A, *Uncaria* alkaloids, and guggulsterone in liver cancer cells increased the response to doxorubicin [294-296]. On the other hand, β-caryophyllene oxide (CRYO), a natural sesquiterpene, enhanced sorafenib cytotoxic effect by inhibiting MDR1, MRP1, and MRP2 in gastrointestinal cancer cells [297, 298]. Moreover, a phenylfurocoumarin derivative that interacts with the substrate binding site of BCRP increased the sensitivity of colon cancer cells to irinotecan [299].

The search for chemosensitizers within drugs already approved for repurposing continues. For example, clarithromycin and sildenafil are potential MDR1 inhibitors [300], whereas metformin can inhibit MRP2 [301].

Combining therapies

Another approach to improve treatment response is the co-administration of conventional antitumor agents and other drugs with more selective mechanisms of action. This strategy might also allow dose reduction of each drug and, hence, decrease adverse reactions, which will ultimately impact on the patients' outcome [302].

In this regard, TKIs are examples of molecular targeted therapy that block tumor growth and its spread, as they act directly on signaling pathways involved in cell cycle, cell death, metastasis, and angiogenesis [303, 304]. TKIs have greater selectivity and lower toxicity than other non-targeted drugs [305]. Their combination with adjuvant therapies was proposed to overcome drug resistance to chemotherapeutic regimens [222, 306].

Interestingly, several TKIs have shown potential as chemosensitizers, mainly by inhibiting MDR1, MRPs and BCRP. However, not all of them are substrates of these transporters acting as competitive inhibitors [119, 222]. Specific mechanisms by which TKIs reverse the MDR phenotype remain to be elucidated [305]. TKIs could interact with the substrate-binding sites of ABC transporters, interfering with their transport function without affecting their expression [307].

For example, apatinib, which binds to and inhibits the efflux of MDR1 and BCRP, reversed resistance to doxorubicin and mitoxantrone *in vitro* [307]. Sunitinib, which inhibits BCRP, also reversed resistance to conventional chemotherapeutic agents in breast cancer cells [308].

Blocking ABC pumps' function

Monoclonal antibodies targeting ABC transporters have also been developed, like MRK-16, MRK-17, and UIC2, which target MDR1 and significantly inhibit its function *in vitro* and *in vivo* [309-311].

Lipid membrane-coated carbon nanoparticles, targeting the mitochondrial pyruvate pathway, increased the production of reactive oxygen species (ROS) under the illumination of near-infrared radiation. Consequently, ATP is not available to be utilized by the ABC transporters, so they could be employed to deliver antitumor drugs while reducing efflux pump activity [312].

Some compounds, like protein kinase D inhibitors, were used to increase HepG2 cell sensibility to cisplatin and carboplatin through modulation of proteins belonging to ATP7 family, as these transporters require phosphorylation mediated by this kinase for their activation and trafficking [313].

Modulating ABC pumps' expression

Regulating ABC transporter expression with advanced molecular technology (such as RNA interference or antisense oligonucleotides) is a novel alternative proposed to overcome multidrug resistance [314].

RNA interference (RNAi) can be used to bind and inhibit the transporter's mRNA. For example, microRNA miR-298 could reduce the MDR1-mediated MDR phenotype in breast cancer cells [315]. Synthetic small interfering RNAs (siRNAs) targeting ABC transporter genes, like *ABCC4* in gastric cancer, are being designed and studied [316]. HepG2 cells treated with *ABCC2* antisense construct reverted resistance to doxorubicin, vincristine, cisplatin, and etoposide [317]. Furthermore, RNAi could be introduced in a nanocarrier in combination with an antitumor drug to obtain a better response and improve efficacy [318]. Nanoparticles loaded with doxorubicin and cholesterol-ABCB1-siRNA showed interesting results in a liver tumor model [319]. Transkingdom RNAi (tkRNAi) is another technology in which a nonpathogenic bacterium is used to deliver short hairpin RNA molecules into resistant cancer cells [320]. The anti-ABCB1 shRNA expression vector carried by *E. coli* resulted in significant inhibition of MDR1 expression in several types of human cancer cells and subsequent increase in chemotherapeutic drug concentrations [321].

Gene manipulation is another valid strategy to minimize or abolish drug resistance [314]. RNA-guided "clustered regularly interspaced short palindromic repeats" (CRISPR) gene

editing technology can be an excellent tool to silence cancer-related genes in tumor cells [322]. For example, reducing *ABCB1* expression restored doxorubicin sensitivity in ovarian cancer cells [323]. However, studies are mostly limited to *in vitro* models as the application of such technology in patients is still questioned [324].

Aptamers are small, single-stranded RNA or DNA oligonucleotides (20-60 nucleotides) that bind target molecules with high affinity and specificity [325]. Soldevilla *et al.* synthesized an aptamer to target MRP1 in resistant tumors through a novel approach known as systematic evolution of ligands by exponential enrichment (SELEX) [326].
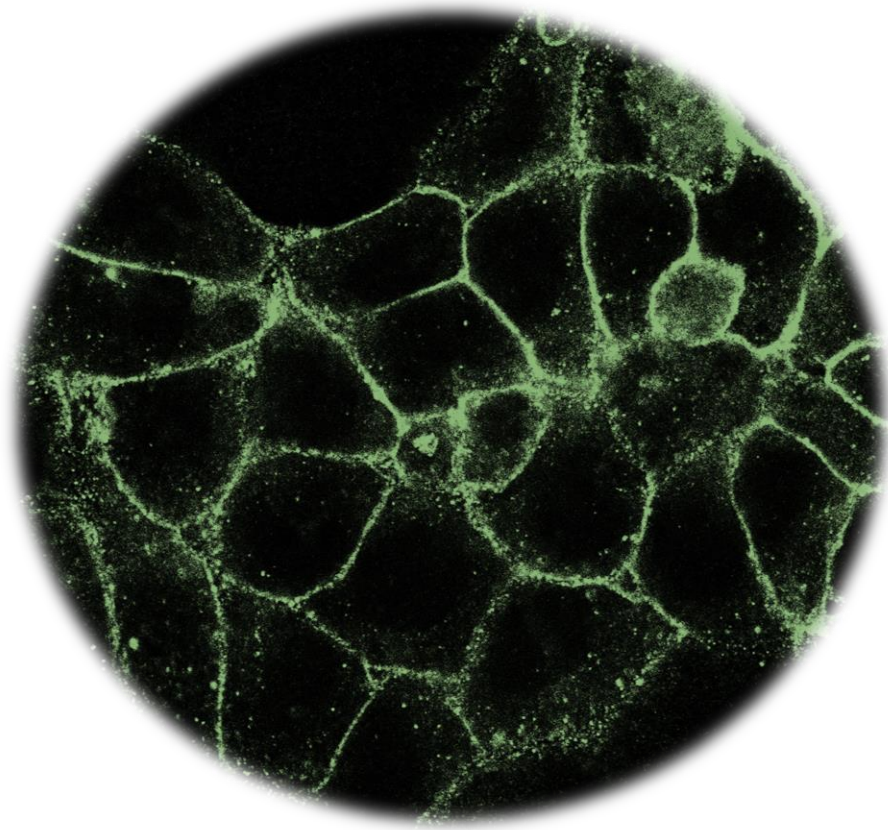
3. MATERIAL AND METHODS

3.1. BIOLOGICAL MATERIAL

3.1.1. Human samples

Paired tumor and adjacent non-tumor samples from patients with HB (n=21) were supplied in the framework of a collaborative project by Dr. Carolina Armengol, Pediatric Hepatic Oncology Group, from Germans Trias i Pujol Institute (IGTP), Badalona (Spain). All patients or their legal representatives signed informed consent for the use of tissue samples in biomedical research. Patients' data were kept confidential and safe in accordance with European Union guidelines. The Clinical Research Ethics Committee of Salamanca approved the research protocol (January 8th, 2016). Relevant demographic and clinical information of these patients included in the study is shown in **Table MM1**.

Table MM1. Demographic and clinical information.

Parameter or variable	Values
Age, months (mean, [range])	20.7, [4-65]
Sex (Male/Female)	13/8
Serum AFP, ng/ml (range)	18×10^3 - 2.19×10^6
Histology (Epithelial/Fetal/Mixed/Regression)	6/1/13/1
Tumor stage	
PRETEXT stage (I/II/III/IV)	1/7/8/5
Metastasis at diagnosis (Y/N)	3/18 (14.3%)
Multifocality (Y/N)	7/14 (33.3%)
Vascular invasion (Y/N)	5/16 (23.8%)
Neoadjuvant chemotherapy (Y/N)	21/0 (100%)
SIOPEL protocol (3/4/6)	6/5/10
Follow-up, months (mean, [range])	43.9, [3.5-96.5]
Died of the disease	4.8%
Molecular classification of the tumor	
16-gene signature (C1/C2)	10/11
Molecular stratification risk (E1/E2/E3)	9/5/7

Neoadjuvant chemotherapy consisted of cisplatin and doxorubicin.
AFP, alpha-fetoprotein; PRETEXT, pretreatment extent of disease.

3.1.2. Animal models

Athymic nude mice (Ico:*Swiss-nu/nu*) (Charles River Laboratories, Barcelona) were used for *in vivo* chemosensitization studies. The animals were maintained in a sterile environment with controlled temperature (20-25°C), relative humidity (40-70%), a

constant light/dark cycle (12 h/12 h) and received food and drink (commercial granulated feed) *ad libitum*.

The animals were always treated following the specifications of the European Community (2010/63/EU and 2003/65/EC) and the current Spanish regulations for the use and care of animals for experimentation (RD 53/2013, BOE 34/11370-421, 2013). The protocols were approved by the Bioethics Committee for Animal Experimentation of the University of Salamanca (October 10th, 2016) and the Consejería de Agricultura y Ganadería, Junta de Castilla y León (November 2nd, 2016).

3.1.3. Cell lines

The following cell lines were used:

- Provided by the American Type Culture Collection (ATCC, LGC Standards, Barcelona): HepG2, derived from human hepatoblastoma (HB8065).

- Provided by Dr. Carolina Armengol: HuH6 cells, derived from human hepatoblastoma.

- Provided by XenTech (Évry, France): cell lines obtained from patient-derived xenographs HB-214, HB-282 and HB-303.

3.2. CHEMICAL COMPOUNDS

The following solid compounds have been used: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (AppliChem, Madrid); anhydrous sodium acetate, D-glucose, diethyl pyrocarbonate (DEPC), disodium tartrate, ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydroxide, sodium pyruvate, trishydroxymethyl aminomethane base (Trizma) or its hydrochloride (Tris-HCl) (Sigma-Aldrich, Madrid); calcium chloride, disodium phosphate, magnesium chloride, magnesium sulphate, potassium chloride, potassium dihydrogen phosphate, potassium hydrogen phosphate, sodium carbonate (VWR International, Barcelona).

The following organic solvents have been used: β-mercaptoethanol, dimethyl sulfoxide (DMSO), ethanol, formaldehyde, formic acid, glycerol, isopropanol, methanol (Sigma-Aldrich); glacial acetic acid, hydrochloric acid (VWR International).

The compounds listed in **Table MM2** were used as fluorescent substrates or as inhibitors of proteins of interest. Propidium iodide (PI) (Sigma-Aldrich) was used for its fluorescent properties and its ability to label dead cells.

Table MM2. Substrates and inhibitors of the indicated transporters.

Transporter/s	Type	Name	Brand
MDR1	Substrate	Rhodamine 123	Sigma
	Inhibitors	Elacridar	Selleckchem
		Tariquidar	Selleckchem
		Zosuquidar	Selleckchem
		Verapamil	Sigma
MRP1, MRP2	Substrate	CAM	Thermo
	Inhibitors	MK-571	Sigma
		Probenecid	Sigma
MRP3, MRP4, MRP5	Substrate	CF	Sigma
	Inhibitor	Diclofenac	Sigma
BCRP	Substrate	Mitoxantrone	Sigma
	Inhibitor	FTC	Sigma

CAM, Calcein acetoxymethyl ester; CF, 5(6)-carboxyfluorescein diacetate; FTC, fumitremorgin C.

Besides, different antitumor drugs, other medicinal and natural compounds, described in **Tables MM3, MM4,** and **MM5**, were employed. Idarubicin (ZAVEDOS®) and one of the TKIs (CCL-22) were provided by the Pharmacy Service of the University Hospital of Salamanca.

Table MM3. Compounds with natural origin.

Name	Type	Brand
CCL-25	Polyphenol	Sigma
CCL-26	Terpene	Sigma
CCL-27	Flavonoid	Sigma
CCL-28	Flavonoid	Thermo Fisher

Table MM4. Other medicinal compounds.

Name	Type	Brand
CCL-29	Analgesic	Sigma
CCL-30	NSAID	Sigma
CCL-31	NSAID	Sigma
CCL-32	NSAID	Thermo Fisher
CCL-33	NSAID	Thermo Fisher
CCL-34	Statin	Selleckchem
CCL-35	Statin	Thermo Fisher
CCL-36	Statin	Thermo Fisher
CCL-37	Statin	Selleckchem
CCL-38	Statin	Selleckchem
CCL-39	Statin	Selleckchem
CCL-40	Statin	Thermo Fisher

NSAID, non-steroidal anti-inflammatory drug.

Table MM5. Antitumor drugs used.

Name	Type	Brand
5-Fluorouracil	Pyrimidine analog	Sigma
CCL-1	TKI	Selleckchem
CCL-2	TKI	Sigma
CCL-3	TKI	Sigma
CCL-4	TKI	Selleckchem
CCL-5	TKI	Selleckchem
CCL-6	TKI	Selleckchem
CCL-7	TKI	Santa Cruz
CCL-8	TKI	Selleckchem
CCL-9	TKI	Sigma
CCL-10	TKI	Selleckchem
CCL-11	TKI	Sigma
CCL-12	TKI	Sigma
CCL-13	IDH1 inhibitor	Selleckchem
CCL-14	TKI	Sigma
CCL-15	TKI	Santa Cruz
CCL-16	TKI	Selleckchem
CCL-17	TKI	Sigma
CCL-18	TKI	Sigma
CCL-19	TKI	Selleckchem
CCL-20	TKI	Selleckchem
CCL-21	TKI	Selleckchem
CCL-22	TKI	UHS
CCL-23	TKI	Sigma
CCL-24	TKI	Selleckchem
Cisplatin	Pt derivative	Sigma
Doxorubicin	Anthracycline	Sigma
Epirubicin	Anthracycline	Sigma
Etoposide	Podophyllotoxin	Sigma
Idarubicin	Anthracycline	ZAVEDOS®
Irinotecan	Camptothecin	Sigma
Oxaliplatin	Pt derivative	Thermo Fisher
Vinblastine	*Vinca* alkaloid	Sigma
Vincristine	*Vinca* alkaloid	Sigma

TKI, tyrosine kinase inhibitor; UHS, University Hospital of Salamanca.

MRP1 and MRP2 inhibitors were obtained through MolPort (NY, USA), Cymit Quimica (Barcelona, Spain) or Selleckchem (Planegg,Germany) depending on availability.

The products we used to perform molecular biology techniques were oligonucleotide or primers (Biomers.net, Ulm, Germany, and Isogen, De Meern, The Netherlands), microfluidic cards (TLDAs), "SYBR Green I PCR Master Mix", "Taqman® Universal PCR

Master Mix" kit, "illustra RNAspin Mini RNA Isolation kit"; "High-Capacity cDNA Reverse Transcription" kit (Thermo Fisher Scientific).

The following products were used for protein detection: freeze-dried skimmed milk (AppliChem); bovine serum albumin (fraction V), bromophenol blue, Folin-Ciocalteu reagent, paraformaldehyde, protease inhibitor cocktail (Sigma-Aldrich), acrylamide, ammonium persulphate, nitrocellulose membranes, tetramethylethylenediamine (TEMED), Tris buffers (0.5 M, pH 6.8 and 1.5 M, pH 8.8) and protein molecular weight markers "Dual Color" (Bio-Rad, Madrid) and "Page Ruler" (Thermo Fisher Scientific), as well as Whatman paper (Whatman, Madrid); Enhanced ChemoLuminiscence (ECL) detection reagents (GE Healthcare); OptiplusTM slides (Menarini Diagnostics, Barcelona); Entellan® mounting medium, Tissue-Tek® "Optimum Cutting Temperature", and Tween® 20 (VWR International); mounting medium for immunofluorescence (Dako Diagnósticos, Barcelona) and 4,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific). HRP peroxidase-conjugated secondary antibodies (GE Healthcare) and fluorescent labelled with AlexaFluor® 594 or AlexaFluor® 488 (Thermo Fisher Scientific) (mouse, goat, rabbit, and rat anti-IgG) were also used.

Culture media "Dulbecco's Modified Eagle's Medium (DMEM)-high glucose", and "Minimum Essential Medium (MEM)", trypan blue, trypsin from porcine pancreas (Sigma-Aldrich); advanced DMEM/F12, antibiotic mixture (penicillin/streptomycin) and antifungal (amphotericin B), fetal bovine serum (FBS) and GlutaMAX™ glutamine supplement (Thermo Fisher Scientific) were employed for cell culture.

Another product used for *in vitro* experiments was FACS Flow (flow cytometry enveloping fluid) (BD Biosciences, Madrid).

Forane® (isofluorane inhalation anesthesia) and Nembutal NR or sodium pentobarbital (Abbot Laboratory, Madrid) and Matrigel™ (BD Biosciences, USA) were employed for animal experiments.

Other reagents were of analytical grade and readily available from commercial sources.

3.3. EQUIPMENT

MiliQ Integral 3 System (Millipore-Merck, Madrid) purified water to obtain both distilled and ultrapure water.

Weighing was performed on Precisa 125 A, Precisa 600 C and Sartorious BL 150S digital electronic scales (Taper Group, Madrid).

pH was determined with a Crison PH BASIC 20 electrode (Crison Instruments, Barcelona).

An autoclave "Presoclave-III 80 L" 4001759 (JP Selecta, Barcelona) was used to sterilize the material, while cell culture solutions were sterilized by vacuum filtration with 0.1 and 0.2 µm Steritop™ filters and a Millipore pump XX5522050 (Millipore, Madrid).

Small equipment, such as Gilson micropipettes, Stratagene picofuge (VWR International Eurolab), vortex (Prosisa, Salamanca), Thermomixer (Eppendorf, Hamburg), and magnetic stirrers (Ika Works, USA), was used.

Refrigerated centrifuges 5417R and 5810R (Eppendorf, Hamburg) were used.

Absorbance measurements were performed with a Hitachi U-2000 spectrophotometer (Taper Group) for individual samples or with a microplate reader for 96-well plates, models ELx808 (BioTek) and Multiskan SkyHigh (Thermo Fisher Scientific).

For spectrophotometric assessment of RNA concentration, a NanoDrop 1000 (Thermo Fisher) and its corresponding software were used.

A LAUDA Ecoline StarEdition RE106 thermostatized bath and a QBA2 Grant thermoblock (Thermo Fisher) were used for incubations at specific temperatures.

Quantitative real-time PCR was performed on the ABI PRISM® 7300 Sequence Detection System or Quant Studio® 3 (Applied Biosystems) in individual 0.2 ml tubes.

The ABI PRISM® 7900HT Sequence Detection System was used for TaqMan Low Density Array (TLDA).

Acclaim column Pepmap RSLC C18 (75 µm ID × 150 mm, 3 µm, Thermo Fisher Scientific), QTRAP 6500+ triple quadrupole mass spectrometer QExactive Plus (Thermo Fisher Scientific), XcaliburTM software (Thermo Fisher Scientific) and TraceFinderTM (Thermo Fisher Scientific) were used by the Signatope GmbH (Germany) for specific protein detection.

A Mini-Protean II Cell system with a PowerPac 300 power supply was used for protein electrophoresis and a Trans-Blot Cell II system (Bio-Rad) for electrophoretic transfer to nitrocellulose membranes.

Fujifilm LAS-4000 image analysis equipment (TDI, Madrid) allowed the detection of the luminescence of the western blot membranes as well as the Chemidoc MP equipment (Bio-Rad). Images were taken with a cooled camera, without filters and with a lens aperture of f0.8 (for chemiluminescence). Image analysis was performed with MultiGauge v3.0 software (Fujifilm) or Image Lab (Bio-Rad) depending on the equipment used.

Tissue sections were made with a Leica Microsystems 1900UV cryostat (Barcelona) or a microtome for paraffin-embedded samples.

The Nikon Eclipse TE 2000-S microscope (Nikon, Duerolab) was used for cell culture monitoring and the confocal microscope model TCS SP2 (Leica), from the Cancer Research Institute (Salamanca), was used for immunofluorescence imaging.

Cell cultures were handled under sterile conditions using a laminar flow hood model AV-30/70 (Telstar, Barcelona) in a Hepa Class 100 thermostatised incubator (Thermo Fisher) in culture plates and TPP Roux flasks (Fisher Sientific, Madrid). Cryotubes of the different cell lines were preserved in a liquid N_2 tank (Thermo Fisher Scientific). Thoma chambers were used for counting.

The HoloMonitor® M4 live cell imaging system (Phase Holographic Imaging PHI, Sweden) was used to adquire cell migration and proliferation data in real time by phase holographic imaging, with the App Suite software for analysis.

Cell fluorescence was measured by flow cytometry with a FACScalibur model (BD Biosciences), using CellQuestPro software for data acquisition and analysis.

Samples were stored, as appropriate, at -20°C and -80°C in "Forma 900 Series" (Thermo Fisher Scientific) freezers.

3.4. *IN VIVO* EXPERIMENTS

Two subcutaneous implants per mouse (one implant in each flank) of 10^6 wild type HepG2 cells or $2x10^6$ doxorubicin resistant HepG2 cells resuspended in 100 µl Matrigel™ were performed.

When tumors became visible and reached an adequate size (approximately 6 weeks), animals were divided into four groups:

- "Control": mice that received i.p. saline (n=3-4 tumors).
- "Doxorubicin": mice that received i.p. doxorubicin (0,2 mg/kg) twice a week (n=3-4 tumors).
- "Zosuquidar": mice that received i.p. zosuquidar (0,02 mg/kg) twice a week (n= 2 tumors).
- "Doxorubicin + Zosuquidar": mice treated with a combination of the anti-tumor drug and the chemosensitizer (n=3-4 tumors).

Tumor size was monitored twice a week throughout the experimental period. Tumor volume was determined using the following formula:

$$\text{Tumor volume} = \frac{D \cdot d^2}{2}$$

Where "D" represents the long diameter and "d" the short diameter.

The experiment ended with the sacrifice of the mice after 5 weeks from the start of treatment.

3.5. CELL CULTURES

The following culture media were used for each cell type:

- HepG2 (and sublines): MEM supplemented with 2.2 g/L sodium bicarbonate and 110 mg/L sodium pyruvate.
- HuH6 (and sublines): DMEM supplemented with 1% GlutaMAX™.
- HB-PDX (HB-214, HB-282, and HB-303): Advanced DMEM/F12 supplemented with 1% GlutaMAX™.

FBS inactivated at 56°C for 30 min was added to 8% of the culture medium for patient-derived cell lines and 10% in the medium for the rest of the cells. All media were supplemented with 1% of an antibiotic mixture containing penicillin (20 U/ml) and streptomycin (0.02 mg/ml).

The HepG2 medium was further supplemented with an antifungal (amphotericin B 0.05 g/ml).

The following solutions were used:

- Phosphate buffered saline (PBS): NaCl 137 mM, KCl 3 mM, Na_2HPO_4 0.5 mM, KH_2PO_4 1.5 mM (pH 7.4).
- Trypsin solution: 0.25% (w/v) trypsin and 20 mM EDTA in PBS (pH 7.4).

Cell cultures were kept in an incubator at 37°C in an atmosphere containing 5% CO_2 and 80% relative humidity. HepG2-DR cells were cultured on surfaces coated with 0.5 mg/ml collagen type I (BD Biosciences).

For culture passages or seeding cells for experiments, cells were incubated with trypsin and collected in culture medium to inactivate this enzyme. The cells were recovered after centrifuging at 300xg for 5 min. Cell viability was determined by Trypan blue method (section 3.9.1). Finally, the cells were accordingly seeded to the desired confluence on the corresponding supports attending to the experiment.

Cells were resuspended in FBS with 10% DMSO and placed in a MrFrosty™ (Thermo Scientific) to be frozen and stored in liquid N_2. To thaw the N_2-stored cells, the cell-containing cryotubes were placed in a thermostatised water bath at 37ºC. Once thawed, cells were placed in fresh medium, centrifuged 300 xg for 5 min and the pellet was seeded in a culture flask.

3.5.1. Generation of drug-resistant cell lines

To obtain HB-derived cell lines with enhanced resistance to drugs of interest (cisplatin and doxorubicin), HuH6 and HepG2 cells in subconfluence were gradually exposed to increasing drug concentrations. Cell morphology, viability and growth rate were monitored during the process to choose the best moment to increase the drug concentration. In the case of cisplatin and HuH6 cells it was up to 2 μM, and 200 nM for doxorubicin in HepG2 cells. This process lasted approximately 10 months. HuH6-CR cells were continuously cultured in the presence of 2 μM cisplatin and HepG2-DR cells in the presence of 200 nM doxorubicin. The medium with the drug was prepared fresh each time and supplemented with ciprofloxacin (10 mg/l) to prevent bacterial contaminations.

3.5.2. Generation of spheroids

Spheroids are 3D biological models that include cell-cell and cell-extracellular matrix interactions and can be useful to mimic physiological features *in vitro*. Our collaborators at the University of Granada used to obtain them through a modification of the "hanging drop" method, in which the droplets are created by depositing liquid on the lid of a Petri dish and then turning it upside down. This assay is based on cell sedimentation to form aggregates within a droplet, during which time FBS in the culture medium is removed [327].

3.5.3. Cell cultures for studying gene and protein expression

Cells were seeded in 6-well plates at the appropriate density for each cell line (3 ml/well). After the time established for each experiment, the cells were collected and processed to obtain total RNA (section 3.6.1.) or total protein (section 3.7.1.1.), or for transport studies by flow cytometry (section 3.8.).

To determine protein localization by immunofluorescence (section 3.7.3.), cells were seeded at the appropriate density in sterile circular coverslips placed in 24-well plates.

In the case of proteomics studies (section 3.7.1.2.), cell pellets were collected by centrifugation and stored at -80°C until processing.

3.5.4. Cell viability assays

To evaluate the effect of antitumor drugs, cells were seeded at the appropriate density in 96-well plates. After 24 h, the compounds were added, and at 72 h from drug addition, either the formazan (section 3.9.2.) or sulforhodamine B (section 3.9.3.) test was performed. When chemosensitizers were evaluated in combination with antitumor agents, cells were pre-incubated with the inhibitors for 2 h at 37ºC before adding the combination.

To carry out synergy studies, cell viability experiments were performed in a similar way. However, in this case, both compounds were added simultaneously in combination at different doses, starting from 0 until reaching approximately the inhibitory concentration 50 (IC_{50}) of each drug. After 72 h of incubation with the compounds, cell viability was determined using the formazan test (section 3.9.2.).

3.5.5. Cell proliferation assays

Cells were seeded in 24-well plates coated with ibiTreat® polymer (Ibidi, Inycom, Zaragoza) at different cell densities (10000 to 50000 cells/cm^2) to follow the proliferation rate of each cell line.

After 24 h, cells were imaged using a Holomonitor M4 Live Cell Imaging System placed in an incubator at 37 °C and 5% CO_2. Images of 4-5 regions per well were captured every 60 min for 72 h. Time-lapse image analysis was performed with App Suite software. Time-course of cell number and culture confluency compared to the initial time was determined for each region.

3.5.6. Cell migration assays

The wound healing assay was used to study cell migration. Cells were seeded in 24-well plates at the appropriate cell density for each cell line. In the case of the HepG2-derived cell lines, cells were seeded on collagen-coated supports to facilitate their adhesion to the surface and the formation of a monolayer.

24 h after seeding the cells, when they were attached to the plate and have reached the adequate confluence, a wound was made on the cell monolayer with a sterile pipette tip. The plate was placed in the Holomonitor M4 within an incubator at 37 °C and 5% CO_2. At least 5 regions from each wound per well were followed and analyzed for 72 h. Time-lapse image analysis was performed with App Suite software.

3.5.7. Colony formation assays

To assess the ability of the different cell models generated to form colonies, cells were seeded in 6-well plates at a density of 1000 cells/well and maintained in culture for 15 days. Culture medium was changed every 2-3 days. After this time, the culture medium was removed, washed with PBS and the cells were fixed with a 7:1 methanol: acetic acid solution at room temperature for 5 min. Subsequently, the fixation solution was removed, and the cells were stained with 0.05% (w/v) crystal violet in 25% methanol for 15 min.

3.6. mRNA QUANTITATION BY RT-qPCR

3.6.1. RNA extraction

The "illustraRNAspin Mini RNA Isolation" commercial kit, was used to extract total RNA from the cells (section 3.5.3) or HB tissue homogenates. Less than 5 million cells or 30 mg of tissue per column were processed following the supplier's instructions. The precipitate obtained was mixed with a lysis buffer that inactivated the RNases, to ensure the integrity of the RNA during the process. Ethanol was then added to provide the appropriate conditions for RNA retention on the silica membrane of the columns. DNA adsorbed on the membrane was removed by treatment with DNAse I, and purification process was completed by several washing and centrifugation steps. Once extracted, total RNA was quantified spectrophotometrically at a wavelength of 260 nm in the Nanodrop.

3.6.2. Reverse transcription (RT)

Complementary DNA (cDNA) was synthesized from the extracted RNA to be later used as template in the PCR. Up to 2 µg of purified RNA were used as template to synthesize cDNA template for quantitative PCR using the "SuperScript® VILO™ cDNA Synthesis Kit" (for tissue samples) or "High-Capacity cDNA Reverse Transcription" kit (Thermo Fisher Scientific) (for cell samples). Both kits contain optimized recombinant Moloney murine leukemia virus (rMoMuLV) reverse transcriptase and random primers. The reaction was carried out in a thermal cycler with the conditions indicated by the supplier.

3.6.3. Primer design for qPCR

The specific primers for coding sequences for each gene studied in this thesis (**Table MM6**) were designed using the specific mRNA sequences published in the GenBank database (http://www.ncbi.nlm.nih.gov/sites/entrez) as references with the OligoAnalizer 3.1 program (http://eu.idtdna.com/analyzer/Applications/Oligoanalyzer) (Integrated DNA Technologies, Coralville, USA). Primers were designed, whenever possible, between two coding exons to minimize amplification of genomic DNA, contamination of RNA samples and primary transcripts (pre-mRNA). The BLAST tool (http://blast.ncbi.nlm.nih.gov/) was used to test their specificity.

The freeze-dried primers were reconstituted in ultrapure water and quantified by UV spectrophotometry at a wavelength of 260 nm in the Nanodrop before use.

Table MM6. Forward (F) and reverse (R) primers used to determine the expression levels of the genes of interest by RT-qPCR.

Gene	Protein	Primers	Type	Amplicon (bp)	Accession number
ABCB1	MDR1	GCGCGAGGTCGGAATGGAT	F	198	NM_000927
		CCATGGATGATGGCAGCCAAAGTT	R		
ABCC1	MRP1	CCGCTCTGGGACTGGAATGT	F	215	NM_004996
		GTGTCATCTGAATGTAGCCTCGGT	R		
ABCC2	MRP2	TGAAGAGGAAGCCACAGTCCATGA	F	171	NM_000392
		TTCAGATGCCTGCCATTGGACCTA	R		
ABCC3	MRP3	CCAAGTTCTGGGACTCCAACCTG	F	160	NM_003786
		ATGATGTAGCCACGACAATGGTGC	R		
ABCC4	MRP4	TGCAAGGGTTCTGGGATAAAGA	F	141	NM_005845
		CTTTGGCACTTTCCTCAATTAACG	R		
ABCC5	MRP5	CGTGAACTGCAGAAGACTAGAGAGACT	F	128	NM_005688
		GGCACACGATGGACAGGATGA	R		
ABCG2	BCRP	CCCAGGCCTCTATAGCTCAGATCATT	F	161	NM_004827
		CACGGCTGAAACACTGCTGAAACA	R		
ACTB	β-Actin	AGCACAATGAAGATCAAGATCATTGCTCC	F	127	NM_001101.3
		ACTCGTCATACTCCTGCTTGCTGAT	R		
GAPDH	GAPDH	TGAGCCCGCAGCCTCC	F	138	NM_002046
		TACGACCAAATCCGTTGACTCC	R		
HPRT1	HPRT1	GCCCTGGCGTCGTGATTAGT	F	140	NM_000194
		AGCAAGACGTTCAGTCCTGTCCATAA	R		
RHOT2	RHOT2	CTGCGGACTATCTCTCCCCTC	F	151	NM_138769.3
		AAAAGGCTTTGCAGCTCCAC	R		
SLC22A1	OCT1	TGCAGACAGGTTTGGCCGT	F	187	NM_003057
		GCCCGAGCCAACAAATTCTGTGAT	R		
SLC22A3	OCT3	CATCGTCAGCGAGTTTGACCTTGT	F	139	NM_021977
		GTAAATGACGATCCTGCCATACCTGTCT	R		
SLC31A1	CTR1	GGAAATTCTTGCCCAACTAAACCCAG	F	277	NM_001859
		TCCGCCTCCTAGGTTCAAGTGATT	R		
SLCO1B1	OATP1B1	GCATCACCTGAGATAGTGGGAAAAGGTT	F	104	NM_006446
		GGAGTCTCCCCTATTCCACGAAGCATAT	R		
SLCO1B3	OATP1B3	GATTCAAGATGTTCTTGGCAGCCCT	F	136	NM_019844
		CCATCAATTAAACCAGCAAGAGAAGAGGA	R		
SLCO2B1	OATP2B1	GAAGGGCAAGGACTCTCCCTCTA	F	87	NM_007256
		GTCAGGTTTGGTGCAATCTGGACT	R		

bp, base pairs; F, forward; R, reverse.

3.6.4. Quantitative PCR (qPCR)

Real-time quantitative PCR is a modality of analytical PCR that allows the quantification of the amplified product during the reaction, absolutely or relatively, by fluorescence detection. SYBR Green I, a fluorochrome present in the "Select SYBR Green PCR Master Mix" reagent mix, was used [328].

Thermal conditions were: 2 min at 50ºC, 10 min at 95ºC, and 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Analyses were performed in duplicate for each sample in the equipment mentioned in section 3.3.

3.6.5. Microfluidic Cards

Microfluidic cards or TaqMan Low Density Arrays (TLDAs) are plates that allow 384 PCR reactions to be performed at the same time, as each of the wells contains the freeze-dried Taqman probe for the gene of interest. The use of TLDAs has several advantages over conventional PCR: it uses a smaller volume of cDNA, and it is a sensitive, reliable, and fast method.

Different TLDA versions were used to study genes of interest related to chemoresistance. One that allowed measuring 93 genes plus the endogenous controls *18S* rRNA, *GAPDH*, and *ACTB* (four samples per plate) and another one with selected 46 genes plus the endogenous controls *GAPDH* and *ACTB*.

To load the sample into the TLDA, the cDNA obtained in the RT reaction (100 ng/port) was mixed with Taqman Master Mix. One sample was loaded into each of the 8 ports of the TLDA. The plate was then centrifuged twice at 300 xg for 1 min. Finally, it was sealed, and the PCR reaction was performed in the equipment mentioned in section 3.3.

The analysis of the results is described in section 3.6.6.

3.6.6. Relative quantification

Relative quantification is based on the threshold cycle comparison or Ct method. The expression level of a housekeeping gene (*RHOT2*, *GAPDH*, and/or *ACTB*, depending on the type of sample and experiment) is used as an endogenous control for normalization of the tested sample. Thus, the ratio between the Ct of the target gene and the Ct of the endogenous control gene results in a normalized Ct value of the target gene.

Finally, the relative expression ratio (R) was calculated using the following formula:

$$R = 2^{-\Delta Ct},$$

where ΔCt = Ct target gene - Ct internal control gene.

In addition, a sample (human liver) was used as a calibrator to compare measurements of different batches.

3.7. PROTEIN IMMUNODETECTION

3.7.1. Protein quantification

3.7.1.1. Total protein: spectrophotometric method

Protein concentration in cell homogenates was determined using a modification of the Lowry spectrophotometric method [329] based on the reduction of the proteins with copper in an alkaline medium and the subsequent reduction of phosphotungstic phosphomolybdic acid with the treated proteins. Serum bovine albumin was used as a reference standard. Finally, the absorbance values of the reaction product are determined spectrophotometrically at $\lambda = 660$ nm.

3.7.1.2. Specific proteins: Triple X proteomics (TXP)

TXP is an innovative approach for protein quantification developed by our collaborators of Signatope GmbH, located at the Natural and Medical Sciences Institute (NMI) of the University of Tübingen (Germany). The term TXP stands for the three variable amino acids (triple X) of the four that constitute the epitope of each peptide of interest, being the remaining one a C-terminal arginine or lysine. This technique uses LC-MS to detect peptides of interest obtained after trypsin proteolysis of the sample, which reduces its complexity, followed by immunoprecipitation [330]. TXP antibodies are versatile, as they can recognize from dozens to hundreds of peptides of a digested proteome because they recognize these small epitopes, presenting "peptide group specificity" [330, 331]. Thus, the number of antibodies required for analysis is drastically minimized, which makes TXP technique especially interesting to study homologous proteins, such as the efflux transporters of the ABC family [332].

The following solutions were used:

- Ammonium bicarbonate and Chaps (3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate): 50 mM ABC, 0.03% Chaps (w/v) in distilled H_2O (pH 7.4).

- Benzonase (Merck, Darmstadt).
- ELISA Blocking Reagent (EBR) (Roche, Mannheim).
- Formic acid (FA) 1% in distilled H_2O.
- Iodoacetamide (IAA) 0.5 M in distilled H_2O.
- Lysis buffer: 1% (w/v) NP-40, 0.01% (v/v) SDS, 0.15 M NaCl, 0.01 M $Na_2HPO_4 \cdot 2 \cdot H_2O$, 2 mM EDTA in distilled H_2O (pH 7.2).
- Mobile phase A: 0.1% FA, H_2O grade LC-MS.
- Mobile phase B: 80% acetonitrile, 0.1% FA and H_2O grade LC-MS.
- PBS and Chaps, "PBSC": solution of 10x PBS, 0.03% (w/v) Chaps in distilled H_2O.
- Phenylmethanesulfonyl fluoride (PMSF): 200 mM PMSF in ethanol (LC-MS grade).
- Synthetic standard peptides (INTAVIS Peptide Services, Tübingen)
- Tris (2-carboxyethyl) phosphine (TCEP) 0.1 M in distilled H_2O.
- Triethanolamine hydrochloride 200 mM in distilled H_2O (pH 8.5).
- Trypsin 1 mg/ml (Thermo Fisher Scientific) in 50 mM acetic acid.
- TXP antibodies (Pineda Antikörper Service, Berlin) purified at Signatope GmbH (Reutlingen).

To perform these studies, cell cultures pellets were obtained as described in section 3.5.4. First, 130 µg of each sample was processed: proteins were denaturated (disulfide bridges broken) followed by trypsinization. Then, the peptides were labelled with stable isotopes ("heavy peptides") and, later, endogenous peptides ("light peptides") were added to the processed sample before immunoprecipitating to use as reference for quantification in the LC-MS. Finally, a magnet was used to separate these eluates from the magnetic beads, which were later separated by ultra-performance liquid chromatography (UPLC) and detected by LC-MS/MS.

3.7.2. Western blot (WB)

The following solutions were used:

- Loading buffer 4x: 250 mM Tris-HCl base pH 6.8, 9.2% SDS, 10% glycerol, 20% β-mercaptoethanol, 0.08% bromophenol blue.
- Migration buffer: 25 mM Tris-HCl base, 192 mM L-glycine y 1% SDS.
- PBS (pH 7.4).
- Ponceau S solution: 0.1% (w/v) Ponceau S in 0.5% acetic acid.

- RIPA Buffer: 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS dissolved in PBS.
- Transfer buffer: 25 mM Tris-HCl base, 192 mM L-glycine, 1% SDS and 20% methanol.
- Tris buffered saline (TBS): 20 mM Tris-HCl base, 137 mM NaCl (pH 7.6).
- TBS-T: TBS supplemented with 0.1% Tween-20 (v/v).

To obtain total protein cell homogenates, cells were first harvested with trypsin (section 3.5.) and washed with PBS. Subsequently, they were lysed with RIPA solution supplemented with 1 % protease inhibitor cocktail (100 µl of mixture for each million cells) and incubated for 20 min on ice with occasional mixing with vortex. Then the lysate was centrifuged for 20 min at 20,000 xg at 4°C to remove cell debris and the supernatant (cell homogenate) was stored at -80°C until use. Finally, the protein concentration in each lysate was determined using a colorimetric method (section 3.7.1.1.).

Polyacrylamide gels were prepared in Tris-HCl buffer using the "Mini-Protean II Cell" system. First, the separator gel was prepared at 5-12% polyacrylamide (w/v), depending on the molecular size of the protein of interest. Once this gel had polymerized, the concentrator gel (4% polyacrylamide) was prepared and put on top of the previous gel together with the comb. It was left to polymerize at room temperature.

Samples were prepared by mixing the protein homogenate (25-50 µg per well) with 4x loading solution and β-mercaptoethanol (5% v/v). Depending on the recommendations for detection of the protein of interest, this mixture was either boiled for 5 min or not. Samples were loaded onto the gel and vertical electrophoresis was triggered in a cuvette with migration solution at a constant current of 15 mA until reaching the separator gel, where it was increased to 30 mA.

After the electrophoresis, proteins in the polyacrylamide gel were transferred to the nitrocellulose membrane in a cuvette with cold transfer buffer at constant current (250 mA) for 1.5-2 h, while gently mixing with a magnetic agitator.

To check the correct loading and transfer of the samples, a staining with Ponceau Red dye was used to detect the proteins present on the nitrocellulose membrane. The dye was removed by washing several times with distilled water.

Once the transfer was completed and the Ponceau red staining was removed, the non-specific binding sites were blocked with 5% (w/v) skimmed milk dissolved in TBS-T for 1 h under agitation. Subsequently, the membrane was incubated overnight at 4°C with the

corresponding primary antibody (specific against the protein of interest, **Table MM7**) dissolved in 1-5% milk.

Table MM7. Primary antibodies used to detect proteins by Western blot.

Protein	Host	Dilution	Brand	Reference
BCRP	mouse	1:500	Abcam	ab15602
GAPDH	mouse	1:1000	Santa Cruz Biotechnology	sc-32233
MDR1	mouse	1:1000	Thermo Fisher	C219
MRP1	rat	1:500	Alexis/Enzo	alx-801-007
MRP2	mouse	1:1000	Alexis/Enzo	alx-801-016
MRP3	rabbit	1:500	SIGMA	M0318
MRP4	rat	1:1000	Abcam	ab15602
MRP5	rat	1:50	OriGene Technologies	AM3188SU-N
Tubulin	mouse	1:1000	Sigma	T9026

The membranes were then washed three times for 15 min each with TBS-T, incubated for 1 h with the corresponding secondary peroxidase (HRP)-conjugated antibody (1:2000 dilution) and washed three times again with TBS-T. Signal was detected after incubating the membrane with "Enhanced ChemioLuminiscence" (ECL) reagent. LAS-4000 or Chemidoc MP image analysis equipments were used to capture chemiluminescence images for the time required to achieve a detectable signal with the lowest background noise.

3.7.3. Immunofluorescence (IF)

The following solution was used:

- PBS Dulbecco without Ca^{2+} and Mg^{2+} (Biochrom, L182-05) for IF (pH 7.4).

Frozen tissue sections (5 µm) were obtained using a cryostat at -20°C and placed on slides that were stored at -80ºC until they were used to optimize the conditions for detecting each protein. Cells were seeded in coverslips as described in section 3.5.3.

Fixation/permeabilization was performed by incubating the samples for 1 min with methanol at -20°C or, in the case of BCRP labelling, samples were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized with 0.01% Triton x-100 for 4 min. Then, after performing 4 washes with PBS in both cases, blocking was performed with 5% (v/v) FBS dissolved in PBS for 30 min.

60

Subsequently, the samples were incubated for 1 h with the corresponding primary antibodies (**Table MM8**). After three washes with PBS (10 min each), the samples were incubated for 1 h in the dark with the appropriate secondary antibodies (diluted 1:1000) in each case anti-IgG conjugated with Alexa Fluor-488® or Alexa Fluor-594® (rabbit, goat, rat, or mouse) and DAPI to stain the nuclei (diluted 1:5000). Finally, the samples were washed three times with PBS (10 min each). Coverslips were placed on a slide with mounting medium (Dako), while tissue samples on slides were coated with mounting medium and a coverslip was placed on top. Sealing was done with nail polish. The labelling was visualized under a confocal microscope in the Microscopy Service of the Cancer Research Institute of Salamanca.

Table MM8. Primary antibodies used to detect proteins by immunofluorescence.

Protein	Host	Dilution	Brand	Reference
BCRP	mouse	1:50	Abcam	ab15602
MDR1	mouse	1:25	Thermo	C219
MRP1	rat	1:40	Alexis/Enzo	alx-801-007
MRP2	mouse	1:33	Alexis/Enzo	alx-801-016
MRP3	mouse	1:50	Abcam	ab3375
MRP4	goat	1:50	NOVUS	NB100-1471
MRP5	rat	1:100	Acris	AM3188SU-N
Na^+/K^+-ATPase	mouse	1:100	Abcam	ab2871
Na^+/K^+-ATPase	rabbit	1:100	Abcam	ab76020

3.7.4. Immunohistochemistry (IHC)

Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections (5 µm thick) were used. The automated platforms Leica Bond III™ (A. Menarini Diagnostics, San Diego, USA) and BenchMark ULTRA (Ventana Medical Systems, Inc, Tucson, AZ) of the Pathology Department of the University Hospital of Salamanca were used to perform IHC. Briefly, after deparaffinization in an oven at 65°C for 24 hours, the samples were rehydrated in a series of decreasing alcohol solutions, washed with Bond Wash Solution, incubated with antigen retriever at pH 9, and endogenous peroxidase activity was blocked by incubation in hydrogen peroxide (3%). The sections were then incubated with the specific primary antibodies diluted as indicated in **Table MM9** and, after washing with Bond Wash Solution, incubated with HRP-bound secondary antibody. After washing with Bond Wash Solution, they were incubated with diaminobenzidine (DAB) and counterstained with hematoxylin. Finally, dehydration in alcohols of increasing gradation, immersion in xylol and mounting with "DEXPEX" (Merck) was performed.

Table MM9. Primary antibodies used to detect proteins by immunohistochemistry.

Protein	Reference	Host	Dilution	Brand
MDR1	E1Y7B	Rabbit	1:50	Cell Signaling Technology
MRP1	sc-18835	Mouse	1:50	Santa Cruz
MRP2	ALX-801-037	Mouse	1:25	Enzo

The Dot Slide Olympus system (version 2.4) and Olympus Corp. equipment (Tokyo, Japan) of the Comparative Molecular Pathology Service of the Cancer Research Center of Salamanca were used to capture high-resolution images. The images were visualized and selected with OlyVIA software.

3.8. *IN VITRO* TRANSPORT EXPERIMENTS

The following solutions were used:

- "Transport medium": NaCl 96 mM, KCl 5.3 mM, KH_2PO_4 1 mM, $MgSO_4$ 0.8 mM, $CaCl_2$ 1.8 mM, D-glucose 11 mM, and HEPES 50 mM (pH 7.4).
- PBS (pH 7.4), described in section 3.5.

The functionality of ABC proteins expressed in the different HB cell lines was determined by measuring the transport of fluorescent substrates by flow cytometry. For this purpose, cells were trypsinized (section 3.5), washed with PBS, and resuspended on "transport medium".

First, 50 µl of cell suspension was placed in each tube and mixed with 50 µl of fluorescent substrates (**Table MM2**) and incubated at 37ºC for the indicated time. To determine the initial loading, the cell suspension was then diluted either with 900 µl of cold transport medium (to stop transport processes) and fluorescence was measured in the flow cytometer. To determine the ABC-mediated efflux, the cell suspension with 900 µl of transport medium at 37°C alone or containing an inhibitor for each pump (Table MM10). The intracellular substrate content was determined after a further 30 or 60 min incubation at 37°C and expressed as a percentage of the initial load. An argon laser of λ=488 nm and emission detectors FL1 (530±30 nm) or FL2 (585±40 nm) were used as light source. For data analysis, the average fluorescence of the cell population consisting of at least 2,000 events (cells) was recorded.

Table MM10. Fluorescent substrates and typical inhibitors used to assess the functional activity of transporters by flow cytometry.

Protein	Substrate		Inhibitor	
BCRP	Mitoxantrone (MTX)	25 µM	Fumitremorgin C (FTC)	2,5-5 µM
MDR1	Rhodamine 123 (Rho)	0.5-1 µM	Verapamil	10 µM
MRP1-2	Calcein acetoxymethylester (CAM)	20-50 nM	Probenecid MK-571	100 µM 10 µM
MRP3-5	Carboxyfluorescein diacetate (CF)	0.25-1 µM	Diclofenac	50 µM

3.9. CELL VIABILITY DETERMINATION

Different methods were used to quantify the number of live cells in cell culture and proliferation or chemosensitivity assays. In case of the latter, colorimetric methods were used as the absorbance, determined spectrophotometrically, is directly proportional to cell viability.

3.9.1. Trypan blue test

To perform this test, a cell suspension and 0.4 % Trypan blue were mixed in equal proportions. The cells were counted in a Thoma chamber with a microscope. Only living cells can remove Trypan blue dye from inside, as such those cells that were not blue were viable.

3.9.2. Formazan test

It is a method based on the transformation of a yellow tetrazolium salt (MTT) into formazan, a violet-colored insoluble compound, by mitochondrial dehydrogenases. The formazan crystals are solubilized with an acid detergent solution. The amount of formazan produced is quantified using a spectrophotometer.

The following solutions were used:

- Cell culture medium (according to the cell line).
- MTT: 5 mg/ml MTT in PBS (pH 7.4).
- Lysis solution: 10 % (w/v) SDS, 10 mM HCl.

To perform this technique, first the medium (with or without compounds) was removed from the well to then add 110 µl of 0.5 mg/ml MTT diluted in culture medium was added to each well of the plate. The plate was incubated for 4 h at 37°C and then 100 µl/well of lysis solution was added. After 16 h incubation at 37°C in the dark, the color intensity of

the supernatant was determined in a plate reader at λ=595 nm. As a blank, those same reagents were added to wells without cells.

3.9.3. Sulforhodamine B test

It is a method based on the detection of the cellular content of proteins using trichloroacetic acid for fixation and sulforhodamine B, a pink aminoxanthene, for labelling as it binds to basic amino acids under acidic conditions [333]. Thus, it provides an index of cellular protein content which is quantified using a spectrophotometer.

The following solutions were used:

- Acetic acid 1% (w/v) in H_2O.
- Trichloroacetic acid (TCA) 5% (w/v) in H_2O.
- PBS (pH 7.4).
- Sulforhodamine B 0.057% (w/v) in 1% acetic acid.
- Tris 10 mM (pH 10.5).

To carry out this technique, cells were first washed with PBS and then fixed for 1 h at 4°C using cold 5% TCA. The cells were washed four times with distilled H_2O and dried for 90 min at 50ºC. Next, the cells were incubated for 30 min at room temperature with 0.057 % sulforhodamine B for staining. After that, the wells were washed four times with 1 % acetic acid and dried for 90 minutes at 50ºC to remove the excess of stain. Later, 200 µl of Tris solution were added to each well to solubilize the stained sample. The plate was incubated for 16 h at room temperature. After incubation, the color intensity of the supernatant was determined in a plate reader at λ=540 nm. Those same reagents were added to wells without cells to use them as blanks.

3.10. *IN SILICO* STUDIES

To predict the interaction between a compound and a protein by docking, it is necessary to have the information about the 3D structure of the protein of interest besides a library of compounds.

As neither human MRP1 nor MRP2 have been crystalized, their structure needs to be predicted *in silico* using a template. The free online tool Phyre2 was used to confirm which available structure in the PDB was a better fit to achieve a homology model. We used USCF-CHIMERA 1.14 software to model both proteins by homology using bovine MRP1 as template.

The identity between the sequences was checked with the EMBL-EBI online tool Clustal OMEGA.

The library of natural and semi-synthetic compounds was obtained from ZINC database.

The docking studies were performed using Autodock 4.2 Lamarckian Algorithm and Autodock Tools 1.5.7rc1 [334]. Defined docking on MRP1 and MRP2 was performed by covering the drug binding domain. Docking parameters were set to 250 runs and 2,500,000 energy evaluations for each cycle with three independent calculations.

Visualization of the docking poses were performed with VMD (Visual Molecular Dynamics, Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign).

3.11. STATISTICAL ANALYSES

Unless otherwise specified, results were expressed as the mean and standard deviation (SD) or standard error of the mean (SEM).

The statistical significance (p) of the comparison between two means was calculated using Student's t-test for paired or unpaired values, as appropriate. If the comparisons were between more groups, an analysis of variance test (ANOVA) was performed, followed by the Bonferroni multiple comparisons test. Wilcoxon's test and Mann-Whitney's U-test were used for paired and unpaired non-parametric values, respectively.

Data were processed with Microsoft Office Excel or GraphPad Prism 6.0.

Analysis of drug combinations and the corresponding graphs were generated with SynergyFinder web app, version 3.0 (https://synergyfinder.fimm.fi) [335]. To quantify the degree of interaction of the combination, the zero interaction potency (ZIP) reference model was used [336]. This method compares the change in the power of dose-response curves between individual drugs and their combinations, assuming that two drugs that do not interact with each other and undergo minimal changes in their dose-response curves.

CHAPTER I:

CHARACTERIZATION OF THE MECHANISMS OF CHEMORESISTANCE IN HEPATOBLASTOMA. ROLE OF THE TRANSPORTOME
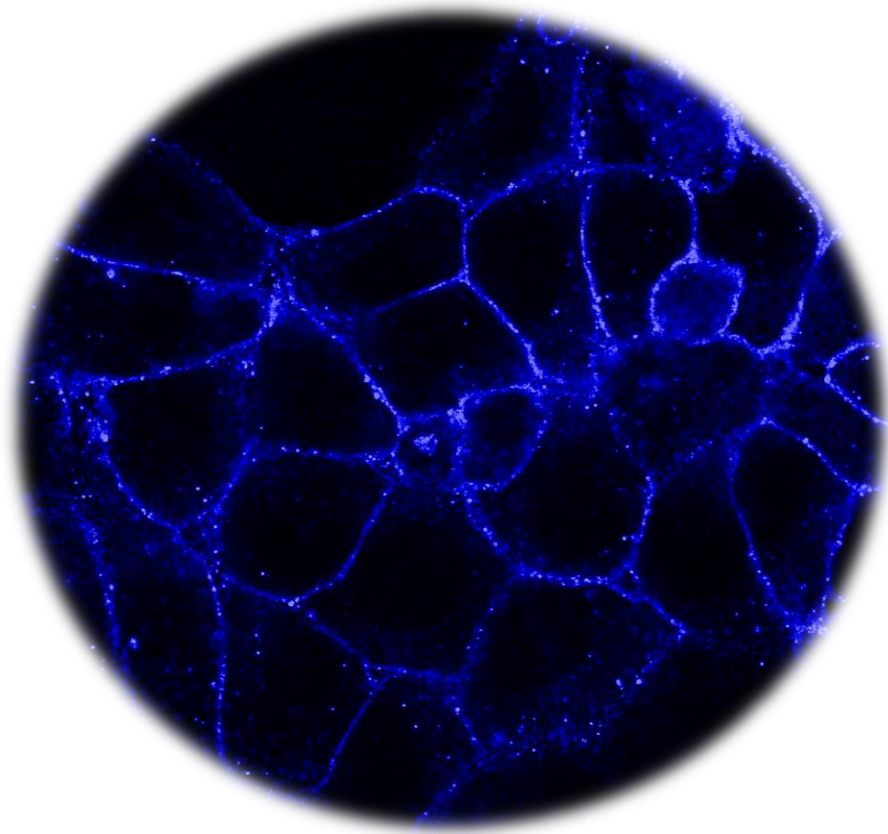
CHAPTER II:

HEPATOBLASTOMA CHEMOSENSITIZATION BY ABC PROTEINS-MEDIATED EFFLUX INHIBITION

6. CONCLUSIONS

FIRST CONCLUSION

Downregulation of the main drug uptake transporters belonging to the SLC superfamily of proteins and upregulation of drug export pumps belonging to the ABC superfamily of proteins may lead to a reduction in intracellular drug concentrations and, therefore, play a role in reducing the response of hepatoblastoma to the pharmacological treatment. Notably, despite the existence of marked interindividual heterogeneity, MRP2 expression levels in hepatoblastoma are associated with a worse response to chemotherapy.

SECOND CONCLUSION

In hepatoblastoma-derived cell lines, the expression profile of genes involved in drug uptake and export is similar to that found in tumors, characterized by low expression of most uptake transporters and high expression of major ABC proteins. Owing to the difficulty of obtaining tumor samples before and after treatment of children with hepatoblastoma, these cell lines were valuable models to study the impact on transportome-dependent chemoresistance and chemosensitivity of this cancer.

THIRD CONCLUSION

The *in vitro* exposure of hepatoblastoma cells to cisplatin and doxorubicin, first-line drugs in treating these patients, induces upregulation of ABC pumps involved in their transport, such as MDR1, MRP1, and MRP2.

FOURTH CONCLUSION

We have generated hepatoblastoma subline cells with a marked multidrug resistance phenotype that are useful models to study the molecular mechanisms underlying the refractoriness to chemotherapy in hepatoblastoma and to search for alternative antitumor pharmacological strategies.

FIFTH CONCLUSION

Although cross-resistance to a panel of anticancer drugs was a shared characteristic of the two chemoresistant hepatoblastoma sublines generated in this study, cabozantinib, a targeted therapy drug used in adult liver cancer, was identified as an effective antitumor drug even in these chemotherapy refractory cells.

CONCLUSIONS

SIXTH CONCLUSION

To overcome the lack of response to chemotherapy, it is possible to sensitize hepatoblastoma cells, even refractory sublines, by manipulating MDR1 function. Thus, zosuquidar, a third generation MDR1 inhibitor, markedly enhances the sensitivity of hepatoblastoma cells to doxorubicin, a substrate of this pump, both *in vitro* and *in vivo*. Moreover, CCL-17 and CCL-24, two drugs that inhibit tyrosine kinase receptors, reduce cell viability, and inhibit MDR1-mediated doxorubicin export, which results in a synergistic antitumor effect.

SEVENTH CONCLUSION

Using *in silico* molecular docking, screening a large dataset of natural and semi-synthetic compounds, followed by *in vitro* confirmatory transport studies, has permitted the identification of several novel MRP1 and MRP2 inhibitors. One of these drugs, the non-toxic flavonoid CCL-45, significantly sensitizes hepatoblastoma cells to antitumor MRP1 and MRP2 substrates, such as doxorubicin and cisplatin.
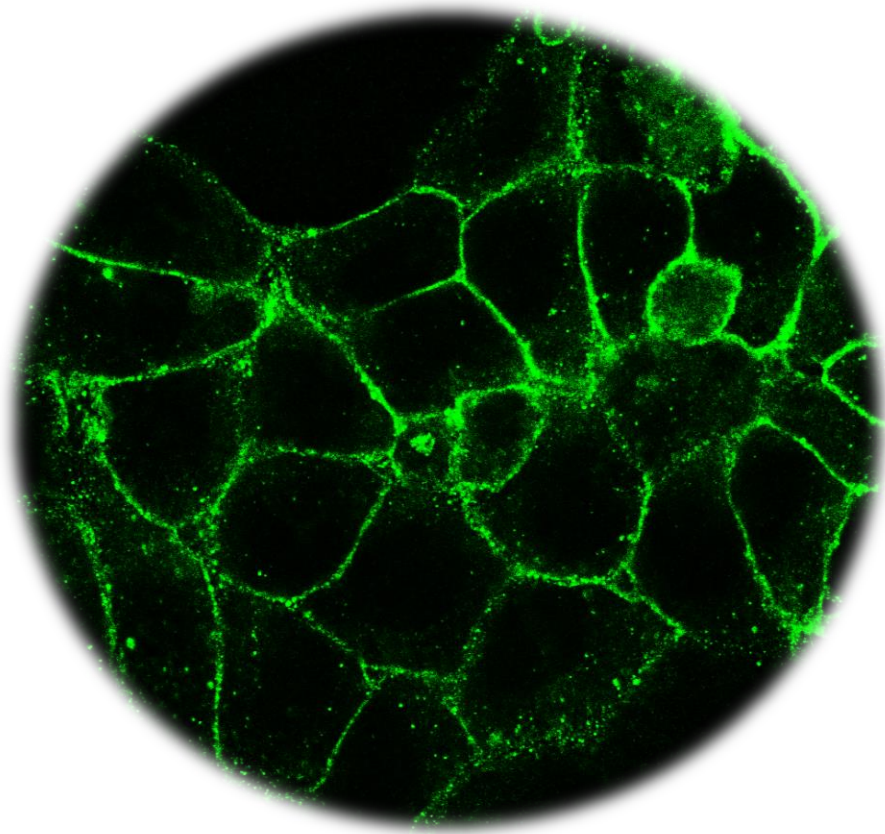
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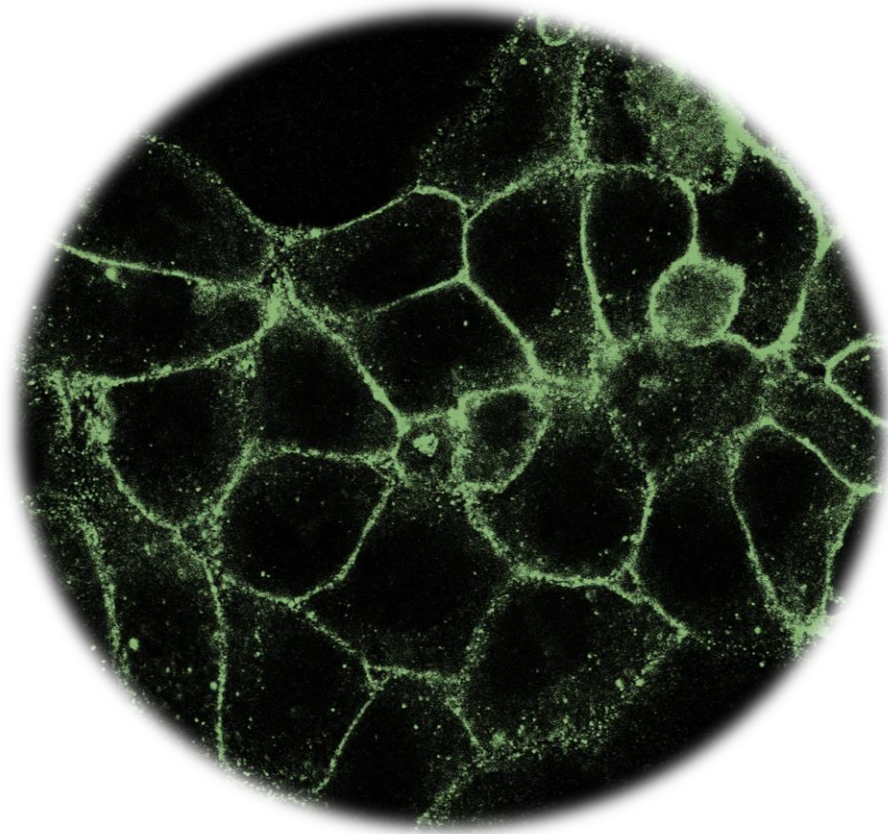
8. RESUMEN

VNiVERSiDAD Ð SALAMANCA

FACULTAD DE FARMACIA

DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA

PAPEL DEL TRANSPORTOMA EN QUIMIORRESISTENCIA Y QUIMIOSENSIBILIZACIÓN EN HEPATOBLATOMA

Memoria presentada por **Dña. Candela Cives Losada** para optar al Título de Doctor por la Universidad de Salamanca

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ABREVIATURAS

ABC	*ATP-binding cassette*
AFP	α-1-fetoproteína
BCRP	Proteína de resistencia del cáncer de mama
CR	Resistente a cisplatino
CTR1	Transportador de cobre 1
DR	Resistente a doxorrubicina
HB	Hepatoblastoma
HCC	Carcinoma hepatocelular
IF	Inmunofluorescencia
IHC	Inmunohistoquímica
MDR	Resistencia a múltiples fármacos
MDR1	Proteína de resistencia a múltiples fármacos 1
MOC	Mecanismo de quimiorresistencia *(mechanism of chemoresistance)*
MRP	Proteína asociada a resistencia a múltiples fármacos
NT	No tumoral
OATP	Polipéptidos transportadores de aniones orgánicos
OCT	Transportadores de cationes orgánicos
PDX	Xenotrasplantes en ratones derivados de pacientes
PRETEXT	*PRETreatment EXTension of the tumor*
qPCR	PCR cuantitativa
SLC	Transportadores de soluto *(Solute Carrier)*
TKI	Inhibidor de tirosina quinasa
TLDA	*TaqMan Low Density Array*
TXP	Proteómica triple X
WB	Western blot
WT	Tipo silvestre (*wild type*)

ANTECEDENTES

El hepatoblastoma (HB) es un tumor maligno de origen embrionario que se desarrolla aún en ausencia de enfermedades hepáticas previas [1]. A pesar de que su incidencia es baja ($2/10^6$ niños al año) [2, 3], ha aumentado en los últimos años [4, 5].

La frecuencia de aparición de mutaciones en HB es muy baja (entre 2 y 3 por tumor) [6], comparada con lo que sucede en el carcinoma hepatocelular (HCC) en adultos [7-10], siendo la menor de entre los tumores pediátricos [11]. Sin embargo, el HB se ha asociado con la activación constitutiva de la vía de señalización Wnt/β-catenina [12] principalmente debido a mutaciones en *CTNNB1* [8], que codifica la proteína β-catenina [10, 13, 14]. Además, un 80 % de los pacientes tienen mutaciones concomitantes de YAP y β-catenina, que son suficientes para generar HB en un modelo animal [15, 16]. En menor proporción, se han detectado mutaciones en *NFE2L2* [6, 8, 17], *PI3KCA*, *IGF* y alteraciones en la vía Hedgehog [6], entre otras.

A nivel histopatológico, los tipos de HB recapitulan diferentes etapas del desarrollo del hígado, incluyendo formas epiteliales, mesenquimales o mixtas [12]. Los tumores fetales bien diferenciados tienen un fenotipo completamente epitelial, mientras que los tumores poco diferenciados, con peor pronóstico, se caracterizan por tener un componente fenotípico predominantemente mesenquimal [18-20].

Para establecer una clasificación molecular de HB se han utilizado resultados de estudios transcriptómicos, epigenéticos o metabolómicos [20-23]. En primer lugar, Cairo *et al* identificaron dos subtipos (C1 y C2) de HB, basándose en el perfil de expresión de 16 genes en el tumor, siendo el subtipo C2 más agresivo [22]. Hooks *et al* subclasificaron este grupo en C2A y C2B atendiendo a la expresión de otros 4 genes [20]. Por otro lado, Carrillo-Reixach *et al* describieron dos subtipos de HB, denominados clúster epigenético A y clúster epigenético B (Epi-CA y Epi-CB) [23], que además estaban fuertemente relacionados con las anteriores subclases moleculares [20, 23]. Sumazin *et al* combinaron el análisis genómico y transcriptómico para identificar biomarcadores de diagnóstico, terapéuticos y de pronóstico, distinguiendo tres subtipos moleculares (HB1, HB2 y HB3) atendiendo al pronóstico [24]. Finalmente, Crippa *et al.* caracterizaron el perfil metabólico de las líneas celulares de HB, observando una elevación de enzimas glicolíticas en HB embrionario [21].

No se ha encontrado una asociación clara entre el desarrollo de HB y la presencia de factores ambientales antes o durante la gestación o enfermedades maternas [25, 26]. Sin embargo, si se ha correlacionado la aparición de este tipo de tumor y el bajo peso

del recién nacido [27]. Se ha visto una predisposición hereditaria a desarrollar HB en una pequeña proporción de casos (menos del 10%) [28], en pacientes con ciertos síndromes, como los de Aicardi y Simpson-Golabi-Behmel [2, 29, 30].

Se suele detectar durante el desarrollo fetal o, más frecuentemente, tras el nacimiento. Los HB congénitos se diagnostican *in utero* o en el primer mes de vida [28]. Tras la exploración física por presencia de signos visibles [6, 28], la detección de cambios en los marcadores bioquímicos como la α-1-fetoproteína (AFP) [28] y el diagnóstico por imagen [6], es necesario un diagnóstico histológico [28].

Se ha desarrollado un sistema para evaluar la extensión del tumor dentro del hígado antes del tratamiento (PRETEXT), que permite la estratificación de los pacientes recién diagnosticados para seleccionar la estrategia de tratamiento más adecuada para cada paciente [31]. Aunque la aproximación terapéutica suele variar entre los países [32], la resección quirúrgica, la quimioterapia basada principalmente en la administración de cisplatino y doxorrubicina pre- y/o postoperatoria, así como el trasplante hepático son las opciones actuales para tratar a estos pacientes [33-35].

La quimioterapia y los avances en las técnicas quirúrgicas han contribuido a alcanzar tasas de supervivencia de hasta el 70-80% en comparación con el 20% obtenido cuando los pacientes se trataban sólo con cirugía [31]. La mayoría de los pacientes con HB responden bien al tratamiento estándar, sin embargo, existe un subgrupo de alto riesgo con mal pronóstico (20% de los casos) [36], que presenta tumores más agresivos caracterizados por una marcada quimiorresistencia [37]. Además, el 12% de los pacientes en remisión completa son propensos a recaer [38]. La combinación de ciertas características clínicas y biológicas podrían utilizarse con valor pronóstico [8, 24, 39].

Esta refractariedad puede deberse a la presencia de un fenotipo de resistencia a múltiples fármacos (MDR) en las células tumorales, conferido por complejos y potentes mecanismos de quimiorresistencia (MOC). Estos pueden estar intrínsecamente presentes en las células tumorales o adquirirse durante el tratamiento. Nuestro grupo de investigación ha clasificado los MOC en siete grupos: una reducción de la expresión/función de los transportadores de solutos (*Solute Carrier*, SLC) presentes en la membrana plasmática, implicados en la captación de fármacos (MOC-1A) o un aumento de la expresión/función de las bombas exportadoras pertenecientes a la superfamilia de proteínas ABC (del inglés *ATP-binding cassette*) (MOC-1B); una menor proporción de fármaco activo frente a inactivo dentro de las células tumorales debido a cambios en las enzimas responsables de la activación de los profármacos o la inactivación de los fármacos (MOC-2); cambios en la expresión/función de las dianas

moleculares de los agentes antitumorales (MOC-3); mayor capacidad de reparación de los daños en el ADN inducidos por los fármacos en las células tumorales (MOC-4); disminución de la expresión/función de los factores pro-apoptóticos (MOC-5A) o aumento de la expresión/función de las proteínas anti-apoptóticas (MOC-5B); cambios en el microambiente de las células tumorales que reducen la eficacia de los fármacos (MOC-6) y adquisición de características de células madre o la transición epitelio-mesenquimal (MOC-7). Los MOCs responsables de la falta de respuesta a la quimioterapia aún no son bien conocidos en HB [40].

En este trabajo nos propusimos profundizar en el estudio del MOC1 para identificar los transportadores de membrana plasmática responsables de la refractariedad a fármacos antitumorales del HB, lo que podría ser de gran utilidad para emplearlos como biomarcadores diagnósticos y pronósticos [40, 41]. Esto es especialmente importante en pacientes pediátricos por su mayor fragilidad y la inmadurez de sus órganos que pueden determinar en gran medida la eficacia de los fármacos antitumorales y sus efectos secundarios [31]. Esta información es también crucial para el desarrollo de nuevas estrategias para el tratamiento del tumor, pues se podrían utilizar como dianas para quimiosensibilizar al HB frente a los fármacos antitumorales [40, 41].

Existen diversas aproximaciones para lograr una mayor respuesta a la quimioterapia utilizando los transportadores como diana terapéutica [40]. Por un lado, si consideramos emplear los trasportadores de captación de fármacos para mejorar los resultados del tratamiento, se pueden realizar modificaciones químicas en fármacos antitumorales para facilitar su reconocimiento por estas proteínas [42-44], encapsular fármacos en nanopartículas [45, 46] o usar terapia génica con vectores virales para incrementar su expresión [47, 48]. Otra alternativa sería utilizarlos para alterar el metabolismo de las células tumorales y, por tanto, inhibir su crecimiento [41, 49].

Por otro lado, si buscamos modular la actividad de los transportadores involucrados en la exportación de fármacos, podemos optar por utilizar compuestos capaces de inhibir las bombas ABC [50, 51], terapias combinadas con fármacos con mecanismos de acción más específicos del tumor, como los inhibidores de tirosina quinasa (TKI) [52, 53], terapia génica para silenciar su expresión, basadas en RNA de interferencia o la tecnología CRISPR/Cas [54-56] o bloquear su actividad mediante anticuerpos específicos o alterando su mecanismo de transporte [57, 58].

OBJETIVOS

El **objetivo global** de esta tesis doctoral fue caracterizar el papel del transportoma en la falta de respuesta al tratamiento farmacológico del HB y, posteriormente, utilizarlo como diana para diseñar estrategias para superar dicha quimiorresistencia.

Para lograr este objetivo, se establecieron varios objetivos parciales.

A) Para determinar el papel del transportoma en el HB, entre otros mecanismos de quimiorresistencia, definimos los siguientes objetivos:

1) Identificación de los transportadores de fármacos implicados en la falta de respuesta del HB a la quimioterapia.

2) Caracterización del transportoma en modelos experimentales *in vitro* del HB, utilizando líneas celulares clásicas y derivadas de pacientes.

3) Determinación del efecto de la doxorrubicina y el cisplatino sobre el transportoma en células del HB tras una breve o prolongada exposición a estos fármacos.

4) Estudio de resistencia cruzada a fármacos utilizados en el tratamiento de segunda línea del HB utilizando líneas celulares quimiorresistentes generadas para este fin.

B) Para desarrollar estrategias de quimiosensibilización en el HB nos propusimos llevar a cabo:

5) Evaluación *in vitro* e *in vivo* de estrategias para mejorar la respuesta a doxorrubicina basadas en compuestos con capacidad de inhibir MDR1 en células derivadas de HB.

6) Búsqueda de inhibidores de MRP1 y MRP2 mediante un estudio *in silico* de *docking* molecular y evaluación *in vitro* del efecto quimiosensibilizante a doxorrubicina y cisplatino de los compuestos seleccionados.

CONCLUSIONES

PRIMERA CONCLUSIÓN

Una menor expresión de los principales transportadores de captación de fármacos pertenecientes a la superfamilia de proteínas SLC y la alta expresión de las bombas de exportación de fármacos, miembros de la superfamilia de proteínas ABC, pueden reducir el contenido intracelular de fármacos antitumorales y, por lo tanto, desempeñar un papel en la refractariedad al tratamiento farmacológico del hepatoblastoma. Así, a pesar de la existencia de una marcada heterogeneidad interindividual, la elevación en los niveles de expresión de MRP2 en hepatoblastoma se asocian a una peor respuesta a la quimioterapia.

SEGUNDA CONCLUSIÓN

El perfil de expresión de los genes implicados en la captación y exportación de fármacos en líneas celulares derivadas de hepatoblastoma es similar al que se encuentra en los tumores, caracterizado por una baja expresión de la mayoría de los transportadores de captación y una alta expresión de las principales bombas ABC. Debido a la dificultad de obtener muestras tumorales antes y después del tratamiento, estas líneas celulares constituyeron modelos valiosos para estudiar la importancia del transportoma sobre la quimiorresistencia y la quimiosensibilidad de este cáncer y su modificación en respuesta a la presión farmacológica.

TERCERA CONCLUSIÓN

La exposición *in vitro* a cisplatino y doxorrubicina, fármacos de primera línea en el tratamiento del hepatoblastoma, produce en células de hepatoblastoma un aumento en la expresión de bombas ABC implicadas en la expulsión de estos fármacos, como MDR1, MRP1 y MRP2.

CUARTA CONCLUSIÓN

Se han generado sublíneas celulares de hepatoblastoma con un marcado fenotipo de quimiorresistencia que son modelos muy útiles para estudiar los mecanismos moleculares responsables de la falta de respuesta a la quimioterapia en hepatoblastoma y para buscar alternativas farmacológicas.

QUINTA CONCLUSIÓN

Aunque la resistencia cruzada a un conjunto de fármacos antitumorales fue una característica compartida por las dos sublíneas de hepatoblastoma quimiorresistentes generadas en este trabajo, el cabozantinib, un fármaco utilizado para tratar el carcinoma hepatocelular, fue eficaz incluso en estas células de hepatoblastoma refractarias a la quimioterapia.

SEXTA CONCLUSIÓN

Para superar la falta de respuesta a la quimioterapia en hepatoblastoma, es posible sensibilizar las células, incluso las sublíneas resistentes, mediante la manipulación de la función de MDR1. De este modo, zosuquidar, un inhibidor de MDR1 de tercera generación, aumenta notablemente la sensibilidad de las células a la doxorrubicina, un sustrato de esta bomba, tanto *in vitro* como *in vivo*. Además, CCL-17 y CCL-24, dos inhibidores de tirosina quinasa, reducen la viabilidad celular e inhiben la exportación de doxorrubicina mediada por MDR1, lo que da lugar a un efecto antitumoral sinérgico.

SÉPTIMA CONCLUSIÓN

El uso de *docking* molecular *in silico* seguido de estudios de transporte *in vitro* de confirmación, ha permitido la identificación de nuevos inhibidores de MRP1 y MRP2 entre numerosos compuestos naturales y semisintéticos. Uno de estos fármacos, el flavonoide no tóxico CCL-45, sensibiliza las células de hepatoblastoma a doxorrubicina y el cisplatino, sustratos de estas bombas.

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