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Comparative response of nine hepatoma cell lines to the four tyrosine kinase inhibitors, sorafenib, lenvatinib, regorafenib and cabozantinib

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Paula Sagmeister

aus

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Ludwig-Maximilians-Universität zu München

Erster Gutachter:	Prof. Dr. med. Enrico de Toni
Zweiter Gutachter:	Prof. Dr. med. Tobias Schiergens
Dritter Gutachter:	Prof. Dr. med. Roland Kappler
ggf. weitere Gutachter:	_____
	_____
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. med. Stefan Michael Munker
Dekan:	Prof. Dr. med. Thomas Gudermann
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ABSTRACT

Introduction: Tyrosine kinase inhibitors (TKI) are the next choice of treatment for advanced hepatocellular carcinoma (HCC) if the preferred gold-standard, checkpoint inhibitors (CPI), are either not feasible or if a progression of disease under CPIs occurred. To date, the use and treatment line of TKIs is solely based on empirical evidence as there are no established biomarkers capable of predicting a specific TKI's response. The aim of this study is to evaluate the differential responsiveness to the four TKIs already approved for clinical use in a cellular model and further investigate their mode of action.

Methods: To this aim, we conducted an *in vitro* study, evaluating the response of nine hepatoma cell lines to sorafenib, lenvatinib, regorafenib and cabozantinib. Hereafter, we conducted a cell cycle analysis of all four drugs on two representative cell lines to evaluate the drugs' interference in the cell lines' cell cycle.

Results: The nine cell lines showed a pronounced variability of response to the four TKIs in a time- and dose-dependent manner. Sorafenib and regorafenib appeared to be homogenously potent on all cell lines, while lenvatinib and cabozantinib induced a more heterogenous pattern of response. All drugs displayed a similar mode of interference in the cell cycle by causing a G1-arrest and/or an increased number of cells going into apoptosis.

Outlook: The diversified responsiveness to the different drugs prompts us to further evaluate potential biomarkers for the four TKIs and question the treatment paradigm for advanced HCCs.

ABSTRAKT (German)

Einführung: Hepatozelluläre Karzinome (HCC) im fortgeschrittenen Stadium werden bevorzugt mit Checkpoint-Inhibitoren (CPI) therapiert. Gibt es Kontraindikationen gegen die Gabe von CPIs oder kommt zu einem Fortschreiten des Tumourgeschehens unter der CPI-Gabe, sind Tyrosinkinase-Inhibitoren (TKI) die nächstfavorisierte Option im Behandlungs-Algorithmus. Aktuell gibt es keine etablierten Biomarker für einen der TKIs, weshalb ihr Einsatz allein auf empirischen Daten beruht. Die Zielsetzung dieser Arbeit ist es, das unterschiedliche Ansprechen auf diese vier bereits zugelassenen TKIs und ihre Wirkungsweise weiter zu untersuchen.

Methoden: Hierfür wurde eine *in-vitro* Studie zum Ansprechen neun verschiedener Hepatom-Zelllinien auf die vier TKIs, Sorafenib, Lenvatinib, Regorafenib und Cabozantinib, durchgeführt. Hiernach erfolgte eine Zellzyklus-Analyse aller vier Medikamente auf zwei repräsentative Zelllinien.

Ergebnisse: Die neun Zelllinien haben eine ausgeprägte Variabilität in ihrem Ansprechen auf die vier Medikamente gezeigt. Während Sorafenib und Regorafenib sich homogen potent zeigten, präsentierte sich das Ansprechen der Zelllinien auf Lenvatinib und Cabozantinib deutlich diverser. Alle Medikamente zeigten einen ähnlichen Mechanismus der Beeinflussung im Zellzyklus einer Zelllinie durch einen G1-Arrest und/oder eine erhöhte Neigung zur Apoptose.

Ausblick: Das breit gefächerte Ansprechen auf die verschiedenen Tyrosinkinase-Inhibitoren bestärkt uns in der Notwendigkeit zur Suche nach Biomarkern für die vier TKIs. Außerdem veranlasst es den empirisch festgelegten Therapiealgorithmus der TKIs für fortgeschrittene HCCs zu überdenken.

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ABBREVIATIONS

AE	adverse event	**IC_{50}**	half maximal inhibitory concentration
AFP	α-fetoprotein	**IGF**	insulin-like growth factor
AKT	Protein kinase B (=PKB)	**MAPK**	mitogen-activated protein kinase
ANOVA	analysis of variance	**mTOR**	mammalian target of raptomycin
ATCC	American Type Culture Collection	**NAFLD**	non-alcoholic fatty liver disease
BCLC	Barcelona Clinic Liver Cancer	**OS**	overall survival
BSC	best supportive care	**PBS**	phosphate buffered saline
CI	Confidence interval	**PD (L)**	programmed cell death protein ligand
CPI	checkpoint inhibitor	**PDGF(R)**	platelet-derived growth factor (receptor)
CTLA	cytotoxic T-lymphocyte-associated protein	**PFS**	Progression free survival
CTNNB1	Catenin beta 1	**PI3K**	phosphoinositide 3-kinases
DMEM	Dulbecco's Modified Eagle's Medium	**PLC**	primary liver cancer
DMSO	dimethyl sulfoxide	**p.o.**	per os
ECOG	Eastern Cooperative Oncology Group	**PR**	proliferation rate
E_{max}	maximal efficacy	**P/S**	penicillin-streptomycin
EpCAM	epithelial cell adhesion molecule	**Raf**	rapidly accelerated fibrosarcoma kinase
ERK	extracellular signal-regulated kinases	**RET**	rearranged during transfection kinase
FBS	filtered bovine serum	**RTK(I)**	receptor tyrosine kinase (inhibitor)
FGF(R)	fibroblast growth factor (receptor)	**SCF(R)**	stem cell growth factor (receptor) = cKIT
Flt	fms-like tyrosine kinase	**SD**	standard deviation
GR	growth rate inhibition	**SS**	steady state plasma concentration
HBs-Ag	HBV surface antigen	**TACE**	Transarterial chemoembolization
HBV	hepatitis B virus	**TGF**	transforming growth factor
HCC	hepatocellular carcinoma	**TKI**	tyrosine kinase inhibitor
HCV	hepatitis C virus	**TNM**	tumour node metastasis
HFSR	hand foot skin reaktion	**TP53**	Tumour suppressor gene 53
HGF(R)	hepatocyte growth factor (receptor) = cMET	**UICC**	Union internationale contre le cancer
HR	Hazard ratio	**VEGR(R)**	vascular endothelial growth factor (receptor)

1. INTRODUCTION

Hepatocellular carcinoma has a continuously increasing incidence rate with approximately 9.5/100,000 cases per year in 2020 [1]. Unfortunately, most HCCs are diagnosed at an advanced cancer stage with medium overall survival of roughly 6-8 months [2]. At this stage of the disease a curative treatment is no longer an option, and the sole intent of therapy is prolongment of life [3]. There are several regimens of treatment which have been approved for clinical use for advanced HCCs: the combination of atezolizumab plus bevacizumab [4], currently considered to be the standard of care in first-line treatment for advanced HCC, and the four TKIs sorafenib [5], lenvatinib [6], regorafenib [7] and cabozantinib [8].

The problem we have been facing for some time now, is that there are no clinically established biomarkers for a specific TKI which can guide us to the most promising treatment option for an individual [9-11]. It is this work's purpose to experimentally compare all four TKIs directly against each other to investigate the TKIs' effect on different hepatoma cell lines and further understand their mode of action.

In addition to writing this thesis, my colleagues and I published the paper "Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis" at Journal of Hepatocellular Carcinoma in 07/2022 [12], in cooperation with another research group at Mannheim University. This work's results, figures, tables, and general thoughts partly overlap with the content of aforesaid paper. The passages in question are cited accordingly and the consent of the publisher for use of figures, tables and ideas has been obtained prior to the submission of this thesis for inspection.

Following this short introduction, the next chapter is supposed to give an overall understanding of HCC, its importance today, the need to further improve the unsatisfactory therapeutic options in the advanced stage, as well as identify problems we are currently working on solving.

2. THEORETICAL BACKGROUND

2.1 Epidemiology and risk factors of HCCs

Primary liver cancer (PLC) is the sixth most common malignant disease with 905,677 new cases in 2020, accounting for 4.7% of all malignant diseases, and the third most frequent cause of cancer related death in the world with 830,180 deaths in 2020, which accounts for 8.3% of all cancer related deaths [1].

PLC is a collective term for hepatocellular carcinoma, cholangiocarcinoma, and other rare tumours, such as mixed hepatocellular cholangiocarcinoma, fibrolamellar hepatocellular carcinoma and the paediatric hepatoblastoma. HCCs account for approximately 90% of all PLC, whereas the rare PLC-subclasses only account for less than 1% [13].

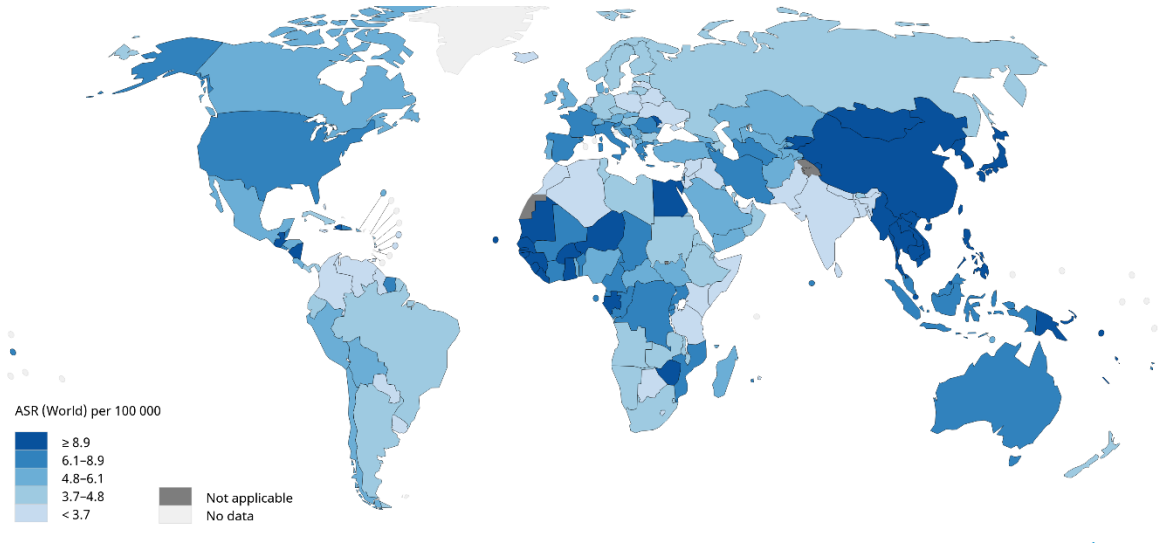

*Figure 1: **Global incidence rate of primary liver cancer.** This map of the world shows the incidence rate of all primary liver cancers per 100,000, estimated for 2020. The darker the blue, the higher the incidence rate. Reprinted with permission from GLOBOCAN 2020, International Agency for Research in Cancer, IARC/WHO. URL: https://gco.iarc.fr/today/online-analysis-map. Accessed on 23.01.2022 [14].*

Figure 1 displays the global incidence rate of primary liver cancer. Approximately 72.5% of all HCC-incidences occur in Asia. Both Eastern Asia and parts of West-Africa show an incidence rate of ≥8.9 cases per 100,000 in both sexes. In comparison, in Central Europe the incidence rates are about half as high, e.g. in Germany there is only 3.7-4.8 cases per 100,000 [1, 14]. In Africa and Asia, the incidence of the disease peaks in 30-50-year-olds, while in Europe and the United States of America, the disease peaks approximately 20 years later, in 50-70-year-olds [15]. The incidence rate for PLC in men is two to four times higher than in women [16].

The biggest risk factors for developing an HCC are liver cirrhosis of any aetiology and chronic hepatitis B (HBV) and C virus (HCV) infections, with or without accompanying cirrhosis. 50% of all HCCs are due to cirrhosis caused by a chronic HBV-infection, and another 25% due to cirrhosis caused by a chronic HCV-infection. In total about 90% of all HCCs are caused by

any form of cirrhosis, one third of every patient with cirrhosis is taken ill with HCC once in their life and 4% of cirrhotic patients get diagnosed with HCC every year [15]. Other known risk factors for HCC include alcohol intake, aflatoxin B1-exposure, non-alcoholic-fatty liver disease (NAFLD), in conjunction with obesity and metabolic syndrome, hemochromatosis, alpha-1-antitrypsin-deficiency, other acquired and inherited metabolic diseases and tobacco smoking [17]. Interestingly, high levels of coffee consumption (>2 cups per day) have been shown to have a decreasing effect on HCC incidences [18].

The Global Burden of Disease 2015 study of primary liver cancer reported a 75%-increase in PLC incidence from 1990 to 2015, stating that this increase may be explained through rising population age, population growth and changing incidence rates [19]. Another study on the age dependent survival benefit for patients with HCC shows that the incidence rate in Germany did not increase over the past 18 years, which might be due to the low prevalence of chronic HBV and HCV infections in Germany [20]. Despite the expected drop in viral hepatitis-related PLC in the future due to the HBV vaccination, highly active treatments of HCV infections [21] and raised awareness for viral hepatitis infections, it is widely believed that the incidence rate for HCCs will come to a steady state, given the simultaneous shift in aetiologies and risk factors in favour of NAFLD, obesity, metabolic syndrome, population aging and growth [17, 22].

2.2 Diagnostics and Surveillance of Liver Cirrhosis

Patients with HCC at an early stage of the disease can complain of missing appetite, weight loss, feeling of pressure in the epigastrium, icterus, ascites, or signs of liver failure. However, there are no distinguishing symptoms at this stage, and all the symptoms named above can also occur in patients with chronic liver disease, which many patients suffer from before developing an HCC, or they can be signs for any other malignancy [15].

Hence, an efficient surveillance for patients at risk is essential to detect small nodules in the liver early and achieve a patient's best possible survival benefit. The method of choice for HCC surveillance is ultrasonography in a 6-month interval, and is indicated for patients with liver cirrhosis, irrespectively of its aetiology, patients with HCV or HBV infection without cirrhosis and patients with non-alcoholic steatosis hepatitis (NASH) [2, 23]. However, there are no screening programs recommended for patients with metabolic syndrome, type 2 diabetes, or obesity without cirrhosis, even though they are arising risk factors for the development of HCCs [24].

If a suspicious nodule in the liver tissue is detected in the ultrasonography screening, a further evaluation using CT or MRI testing is indicated and a biopsy of the mass should be obtained [2, 3].

2.3 Classifications of Staging

A good tumour staging system is essential for predicting an individual's expected survival time and recommending a fitting treatment option. While numeral classifications for HCCs have been introduced over the last couple of years, there are two that stand out, establishing themselves as the standard for HCC classification: The Tumour/Node/Metastasis (TNM)-classification, which classifies the tumour from an anatomical point of view, and the Barcelona Clinic Liver Cancer (BCLC) staging system, which has turned out to be superior to other staging systems in predicting patients' survival outcomes [25].

2.3.1 TNM-Classification

The TNM-classification, as shown in Table 1, is a descriptive, anatomic classification of the expansion of a disease, which was developed by the Union International Contre le Cancer (UICC) and first adopted by the World Health Organisation (WHO) in 1953. It considers the extent of the primary tumour (T), absence or presence as well as extent of regional lymph node metastasis (N) and absence or presence of distant metastasis (M). Further specifications include tumour grading (G1-4) according to Edmondson and Steiner [26], lymphatic (L0 or L1), venous (V0-V2) and perineural invasion (Pn0 or Pn1) and residual tumour classification (R), which describes the extent or absence of tumour remnant after treatment [27].

TNM	Explanation	UICC	
T1	Solitary tumour without vascular invasion	**I**	T1N0M0
T2	Solitary tumour >2cm with vascular invasion or multifocal tumours none >5cm	**II**	T2N0M0
T3	Multifocal tumours at least one of which is >5cm with/without vascular invasion	**IIIA+B**	T3N0M0
T4	Single or multifocal tumour(s) with invasion of adjacent extrahepatic organs or perforation of visceral peritoneum	**IIIC**	T4N0M0
N0	No regional lymph node metastasis		
N1	Regional lymph node metastasis	**IVA**	Any T, N1, M0
M0	No distant metastasis		
M1	Distant metastasis	**IVB**	Any T, Any N, M1

*Table 1: **TNM classification:** T stand for the extent of the primary tumour, N for absence or presence and extent of regional lymph node metastasis and M for the absence or presence of distant metastasis outside of the liver. The combination of these parameters describes the anatomical spread of the tumour. UICC stadiums are a different way to classify the anatomical tumour extent based on the TNM classification system. This table was modified and recreated from Gospodarowicz M, et al. TNM Classification of Malignant Tumours [27].*

Given that the TNM-classification is solely based on the tumour's anatomic expansion and does not consider patients' health status or liver function, it is not suitable to be the basis of further treatment decisions and predict median survival.

2.3.2 Barcelona Clinic Liver Cancer Staging System (BCLC)

The BCLC Staging System, as seen in Table 3, was foremost introduced in 1999 by Llovet et al. and is comprised of tumour extension (size and number of tumours, vascular invasion, and extrahepatic metastasis), degree of liver function impairment as classified by the Child-Pugh Score (see Table 2), patient's general wellbeing and cancer-related symptoms. The patient's well-being is evaluated according to the Eastern Cooperative Oncology Group (ECOG) performance status, which consists of six stages, reaching from no limitations (0) to dead (5) [2].

Points	**1**	**2**	**3**
Albumin-concentration in serum in g/dL	>3.5	2.8-3.5	<2.8
Bilirubin-concentration in serum in mg/dL	<2.0	2.0-3.0	>3.0
Quick in %	>70	40-70	<40
INR	1.7	1.7-2.3	>2.4
Ascites	No	A little	A lot
Hepatic Encephalopathy	No	Grad I-II	>Grad II
Child A: 5-6 points, Child B: 7-9 points, Child C: 10-15 points			

*Table 2: **Child-Pugh-Score:** The Child-Pugh-Score classifies the extent of the liver cirrhosis and therefore the remaining liver function into three groups (A-C), with A being an almost completely functioning and C a severely limited functioning liver. It considers the albumin- and bilirubin-concentrations in the blood, the quick-value, ultrasound diagnosed ascites and hepatic encephalopathy. Table is based on Herold: Innere Medizin [15].*

Additionally, the BCLC-classification relies on the Milan-criteria, which if positive, identify patients most likely to undergo a successful liver transplantation, and least likely to have a recurrence of disease after transplantation. For the Milan criteria to be positive, three criteria need to be fulfilled: firstly, the HCC either consists of one single tumour smaller than 5 cm in diameter or up to three tumours, all of which are smaller than 3cm in diameter, secondly there are no extrahepatic manifestations of the tumour, and thirdly there is no tumorous invasion of blood vessels [28].

BCLC	**ECOG**	**Tumour extension**	**CHILD-Pugh-Score**
0	0	Singular Tumour <2cm or Carcinoma in situ	A or no cirrhosis
A	0	Singular small tumour <5cm or up to 3 tumours <3cm, positive Milan-criteria	A or B
B	0	Multilocular decay, tumour >3cm	A or B
C	1-2	Vascular invasion or metastasis	A or B
D	3-4	Everything higher graded	C

*Table 3: **BCLC classification:** The BCLC staging system divides all HCC into 5 groups (0, A-D) based on well-being of the patient, tumour extension and remaining liver function. BCLC 0 stands for the least advanced HCC with the best survival outcome, while patients with BCLC D have far-advanced tumours with bad liver function and well-being, and an expected survival of a few months. Based on Llovet et al. 1999 [29].*

Using the BCLC classification it is possible to predict a patient's likely survival outcome without having received treatment. Furthermore, it is linked to a treatment algorithm, as can be seen in Figure 2.

2.4 Therapy based on BCLC staging classification

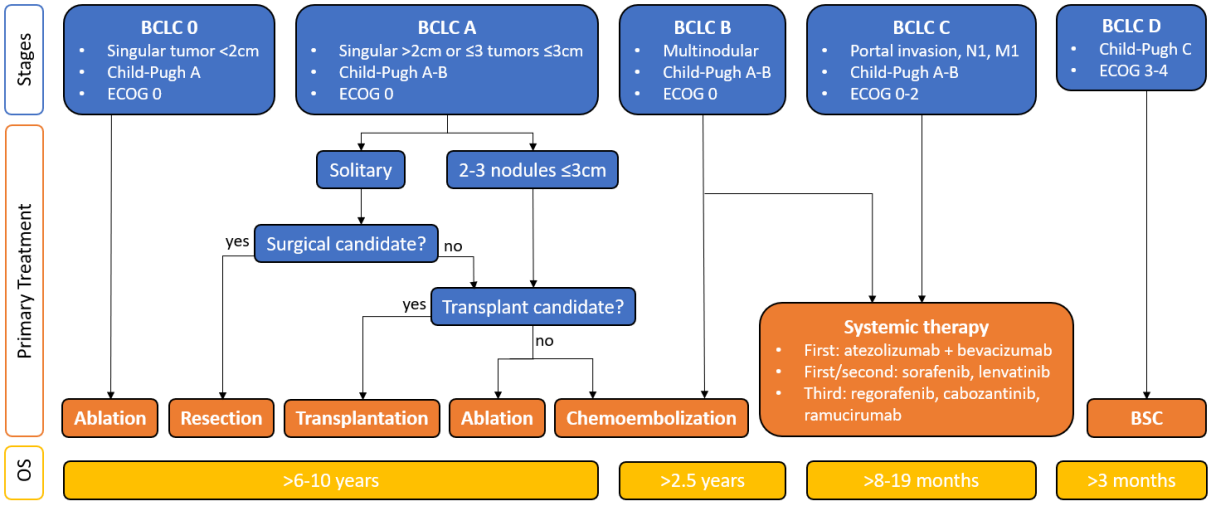

*Figure 2: **Treatment algorithm based on BCLC staging classification.** Patients with HCC can be subdivided into five BCLC stages, 0 and A-D. Depending on the BCLC stage, different treatments are advised and an estimate for survival expectancy can be made. The less advanced the BCLC stage, the better the survival expectancy. Abbreviations: BCLC, Barcelona Clinic Liver Cancer; BSC, best supportive care; ECOG, Eastern Cooperative Oncology Group; M1, distant metastasis; N1, lymph node metastasis; OS, overall survival. This figure was modified and recreated from Forner et al. [30], Vogel et al. [31] and Llovet et al. [3].*

2.4.1 BCLC 0 + A - Very-early and early HCC

BCLC 0 and A are singular tumours <2 cm (0) or >2cm (A) or up to three multinodular tumours all ≤ 3cm (A) with good health status (ECOG-0) and good liver function (no cirrhosis or CHILD-Pugh A). Only 5-10% of all HCCs are classified as BCLC 0 at point of diagnosis. The five-year survival rate after treatment is 80-90% for BCLC 0 and 50-70% for BCLC A. Median survival for patients in BCLC A stage without having received treatment is approximately 36 months [2, 29].

The standard of care for HCCs at BCLC 0 or A stage is a curative treatment approach through tumour resection, liver transplantation, thermal ablation or transarterial chemoembolization (TACE). Alternatively different forms of radiotherapy, such as stereotactic body radiation therapy, brachytherapy or selective internal radiation therapy can be employed [3, 31].

2.4.2 BCLC B - Intermediate HCC

BCLC B stage consists of multinodular asymptomatic tumours without vascular invasion or extrahepatic manifestation with a median survival for untreated patients of roughly 16 months, which can be prolonged to approximately 40 months after standard of care therapy [2, 29].

The standard of care for HCCs at BCLC B stage is TACE. Alternatively, liver transplantation, tumour resection, systemic therapies or radiotherapy can be therapeutic options for some patients [31].

2.4.3 BCLC C - Advanced HCC

Patients with HCC at an advanced stage, thus BCLC C, experience cancer-related symptoms (ECOG 1-2), macrovascular invasion or extrahepatic manifestation with a median OS of 6-8 months. The aim of treatment at this stage of the disease is a prolongment of life [2, 29].

There are various systemic treatment options currently approved for clinical use for advanced HCC, as can be seen in Figure 3.

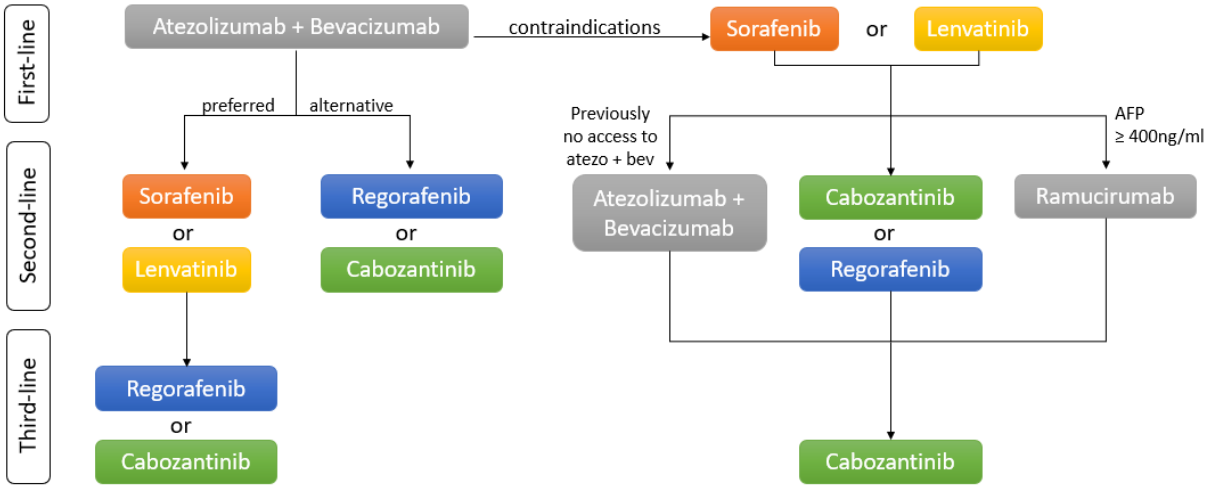

*Figure 3: **Therapy algorithm at BCLC C cancer stage.** Atezolizumab + bevacizumab, sorafenib and lenvatinib are approved for clinical use for advanced HCC as first-line therapies, regorafenib, cabozantinib and ramucirumab as second-line therapies and cabozantinib additionally as a third-line therapy option. This figure is based on information from Gordan et al. [32] and Leitlinienprogramm Onkologie [33].*

2.4.3.1 Molecular pathways of HCC

There are various pathways and genomic alterations capable of inducing liver carcinogenesis. In the instance of gene mutations, the most common ones involve the telomerase promoter, leading to the development of liver fibrosis or cirrhosis. Additional mutations involve the tumour suppressor gene (TP)53 pathway leading to loss of tumour suppressor activity that physiologically promotes apoptosis and cell cycle arrest, and mutations concerning Wnt-pathway components. Other possible mechanisms include epigenetic modifications, copy number variations of genomes such as gain or loss of genetic DNA, viral insertions, or gene arrangements that lead to fusion proteins, as can be detected in fibrolamellar carcinoma [34].

The activation of receptor tyrosine kinases (RTK) through different growth or angiogenetic factors leads to the initiation of the MAPK/ERK and PI3K/AKT/mTOR-pathways, which further promote carcinogenesis by having a regulatory effect on apoptosis, proliferation, cell differentiation, angiogenesis and/or metastasis (see Figure 4). These two pathways are present in approximately 50% of all HCCs and have been identified as key players in liver carcinogenesis. The RTKs or rather their autophosphorylation and activation of the kinases following the

binding of activating factors, are the main target points for TKIs, that have been approved for clinical use for advanced HCCs [34, 35].

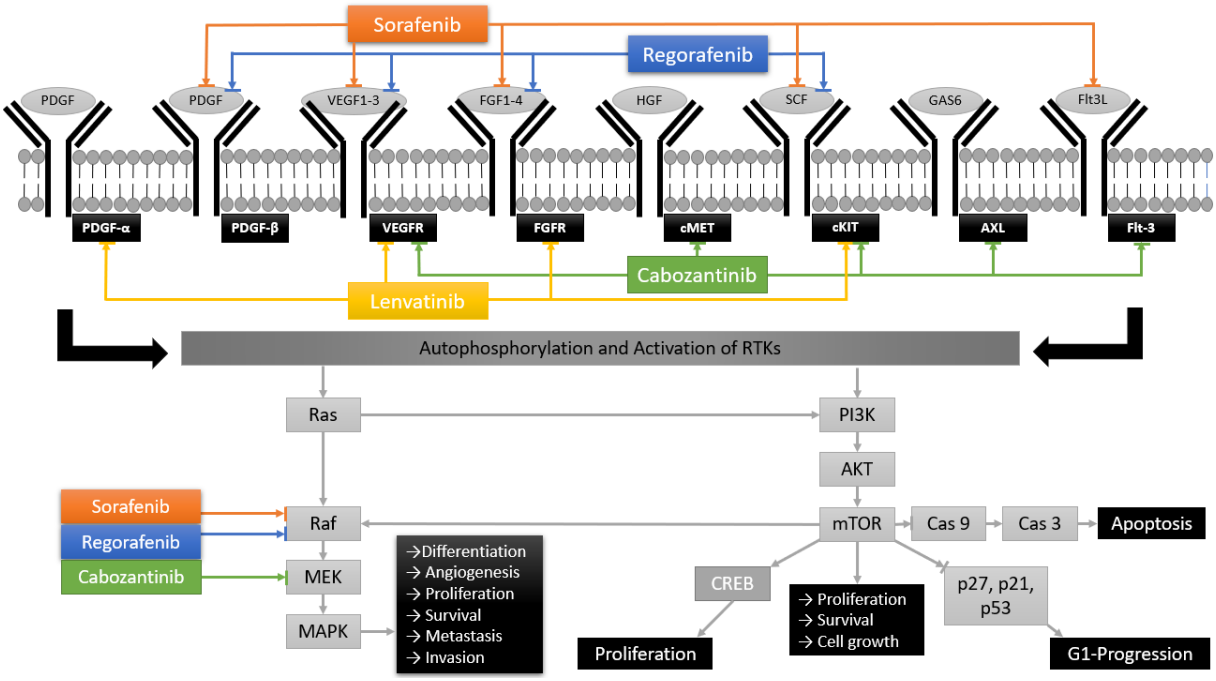

*Figure 4: **Tyrosine kinase inhibitors and molecular pathways of HCC:** Sorafenib, regorafenib, lenvatinib and cabozantinib target various receptor tyrosine kinases by inhibiting their autophosphorylation and activation. This leads to the further inhibition of the MAPK/ERK- and PI3K/AKT/mTOR-pathway, and finally results in the RTK-inhibitors' antiproliferative, antiangiogenic and proapoptotic effect. Abbreviations: AKT (=PKB), protein kinase B; AXL receptor tyrosine kinase, from Greek 'anexelekto', which means uncontrolled; Cas 3/9, caspase 3/9; cKIT (=SCFR), stem cell growth factor receptor; cMET (=HGFR), hepatocyte growth factor receptor; CREB, cAMP response element-binding protein; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; Flt-3, fms-like tyrosine kinase; Flt3L, fms-like tyrosine kinase 3-Ligand; GAS6, growth-arrest-specific-gene 6; HGF, hepatocyte growth factor; MAPK (=ERK), mitogen-activated protein kinases or extracellular signal-regulated kinases; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of raptomycin; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinases; Raf kinase, rapidly accelerated fibrosarcoma kinase; RTK, receptor tyrosine kinase; SCF, stem cell growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor. The RTKs RET and TIE2 (angiopoitin-1-receptor), which are targeted by some RTKI, are not represented in this figure. Modified and recreated from Bangaru et al. [36].*

2.4.3.2 First-line therapy

Trial Name	Randomization	Results	Ref
SHARP	Sorafenib vs. Placebo	Primary Endpoint: OS OS: 10.7 vs. 7.9 months; HR 0.69	[5]
REFLECT	Lenvatinib vs. Sorafenib	Primary Endpoint: OS OS: 13.6 vs. 12.3 months; HR 0.92	[6]
IMbrave150	Atezolizumab + Bevacizumab vs. Sorafenib	Primary Endpoint: OS and PFS OS: NE vs. 13.2 months; HR 0.58 Estimated survival: at 6 months 84.8% vs. 72.2%, at 12 months 67.2% vs. 54.6% PFS: 6.8 vs. 4.3 months; HR 0.59	[4]

*Table 4: **First-line therapies for advanced HCC.** This table shows the three first-line therapies currently approved for clinical use for advanced HCC, as well as the results of their respective phase 3 trials. Abbreviations: HR, hazard ratio; OS, overall survival; PFS, progression free survival.*

The current standard of care first-line therapy for advanced HCC is atezolizumab plus bevacizumab. If there are contraindications against the use of these two agents, such as "a myocardial infarction or stroke within the previous three months, […] a history of autoimmune disease,

[…] therapeutic anticoagulation or […] coinfection with HBV or HCV" [32], the tyrosine kinase inhibitors sorafenib or lenvatinib are alternatively used initials [32].

2.4.3.2.1 Sorafenib

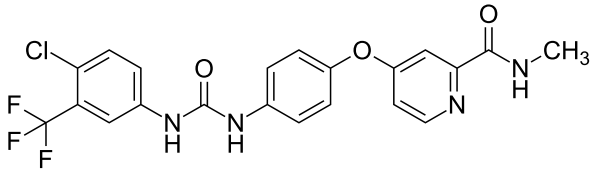

Figure 5: ***Structural formular of Sorafenib.*** *– From Sorafenib, Wikipedia. Accessed from https://en.wikipedia.org/w/index.php?title=Sorafenib&oldid=959243061 [37].*

Sorafenib (Nexavar®, Figure 5), produced by Bayer AG, is a multi-kinase inhibitor which was first approved for the therapy of advanced hepatocellular carcinoma in Europe in 2007, having antiproliferative, antiangiogenic and proapoptotic effects. Its main targets include the inhibition of the rapidly accelerated fibrosarcoma (Raf) serin/threonine kinases Raf-1 and B-Raf, which are the first modulators in the MAPK/ERK cascade, as well as several RTKs, including vascular endothelial growth factor receptor (VEGFR) 1-3, platelet-derived growth factor receptor (PDGFR)-β, stem cell growth factor receptor (SCFR) c-Kit, fms-like tyrosine kinase (Flt-3) and fibroblast growth factor receptor (FGFR)-1(see Figure 4) [38].

Sorafenib is a first-line therapy for patients with HCC in BCLC grades B or C who progressed upon or were unsuitable for locoregional therapies, are in good health (ECOG 0 or 1) and retain sufficiently good liver function (Child-Pugh A). The clinical dose for sorafenib for advanced HCC is 400 mg per os (p.o.) twice daily [2].

The multicentre, phase 3, double blind, placebo controlled SHARP trial, which was conducted in the mid-2000s, investigated the effect of 400 mg sorafenib twice daily in patients with advanced HCC and good liver function (mainly Child-Pugh A). The reported OS of sorafenib vs. placebo was 10.7 months vs. 7.9 months and therefore significantly longer in the sorafenib group. The time to radiological progression was also significantly higher in the sorafenib group (5.5 vs. 2.8 months). The most frequently experienced adverse events (AE) associated with sorafenib are diarrhoea (25-45%), fatigue (20-66%) and hand-foot-skin-reaction (HFSR) (21-45%) [5, 39, 40]. If sorafenib treatment must be discontinued, most commonly due to grade 3 or 4 AEs, tumour progression or liver function deterioration, the survival time is poor [41, 42].

2.4.3.2.2 Lenvatinib

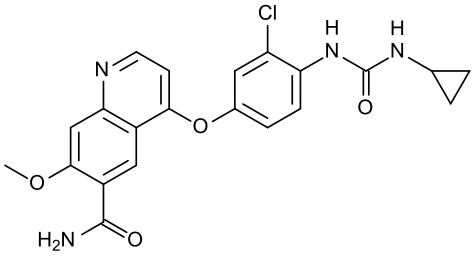

Figure 6: ***Structural formula of Lenvatinib.*** *From Lenvatinib, Wikipedia. Accessed from https://en.wikipedia.org/w/index.php?title=Lenvatinib&oldid=950507198 [43].*

Lenvatinib (Figure 6) is a multi-kinase inhibitor targeting VEGFR1-3, FGFR 1-4, PDGFR-α, RET and c-KIT. In comparison to sorafenib, it also targets FGF-receptors (see Figure 4) [44]. It is indicated as a first line therapy for advanced HCC (BCLC C) with good liver function (Child-Pugh A) and good health status (ECOG 0), or intermediate HCC (BCLC B) which progressed upon or was unsuitable for locoregional therapies, for which it was first approved in Europe in 2019 [2, 45]. Lenvatinib is the preferred first-line therapy for HCCs caused by an HBV-infection, because it is assumed that it will have a more beneficial outcome than sorafenib [44]. The daily dose for lenvatinib is 8mg for patients with a bodyweight <60kg and 12mg for patients who weigh ≥60kg, administered orally once daily [46].

After completion of phase 2 study, which showed that lenvatinib has an effect on advanced HCC and an acceptable safety profile [47], the REFLECT study, an open-label, phase 3, multicentre, non-inferiority trial, was conducted. Lenvatinib in a dosage of 8 or 12mg, depending on a patient's bodyweight, was directly compared to sorafenib 400mg twice daily. In this study, lenvatinib was proven to be non-inferior to sorafenib as a first-line treatment option for advanced HCC, with a median overall survival (OS) of 13.6 months for the lenvatinib group compared to 12.3 months for the sorafenib group, a median time to progression of 8.9 months compared to 3.7 months. The most frequent AEs in the lenvatinib group were hypertension (42%), diarrhoea (39%), decreased appetite (34%) and decreased weight (27%) [6].

2.4.3.2.3 Atezolizumab plus bevacizumab

The anti-programmed death ligand (PDL)1-antibody atezolizumab and the anti-VEGF-antibody bevacizumab were the first to show a significant survival benefit and superiority in the first-line in advanced HCC compared to the long-time favourite sorafenib. After promising results in the phase 1b trial [48] the global, open-label, phase 3 trial IMbrave150 comparing atezolizumab plus bevacizumab against sorafenib in the first-line was conducted. Approximately 500 patients were randomized in a 2:1 ratio receiving either 1200mg atezolizumab plus 15 mg/kg bevacizumab intravenously every three weeks or 400 mg sorafenib orally twice daily.

The primary endpoints were overall survival and progression free survival (PFS). The study showed that OS (estimated survival at 12 months 67.2% vs. 54.6%) and PFS (median 6.8 months [95 confidence interval (CI), 5.7 to 8.3] vs. 4.3 months [95% CI, 4.0 to 5.6]; HR 0.59] were significantly higher in the atezolizumab/bevacizumab group than in the sorafenib group. The most common adverse events caused by atezolizumab-bevacizumab were hypertension (29.8%), fatigue (20.4%), proteinuria (20.1%), aspartate aminotransferase increase (19.5%) and pruritis (19.5%) [4].

Following the results of the IMbrave150 study, atezolizumab plus bevacizumab is now considered the standard of care in the first-line therapy for advanced HCC [31, 49].

2.4.3.3 Second-line therapy

After the preferred first-line therapy with atezolizumab plus bevacizumab, sorafenib or lenvatinib should preferably be used in the second-line, although cabozantinib and regorafenib are also reasonable options. After a TKI in the first-line, regorafenib, cabozantinib, ramucirumab if α-fetoprotein (AFP) ≥ 400 ng/ml, or atezolizumab plus bevacizumab if there was no availability of this combination at point of first-line therapy initiation, are possible second-line treatment options (See Figure 3 and Table 5).

Trial Name	Randomization	Results	Ref
RESORCE	Regorafenib vs. placebo	Primary Endpoint: OS OS: 10.6 vs. 7.8 months; HR 0.63	[7]
CELESTIAL	Cabozantinib vs. sorafenib	Primary Endpoint: OS OS: 10.2 vs. 8.0 months; HR 0.76	[8]
REACH-2	Ramucirumab vs. placebo	Primary Endpoint: OS OS: 8.5 vs. 7.3 months; HR 0.71	[50]

*Table 5: **Second-line therapies for advanced HCC.** This table shows the three second-line therapies currently approved for clinical use for advanced HCC, as well as the results of their respective phase 3 trials. Abbreviations: HR, hazard ratio; OS, overall survival.*

2.4.3.3.1 Regorafenib

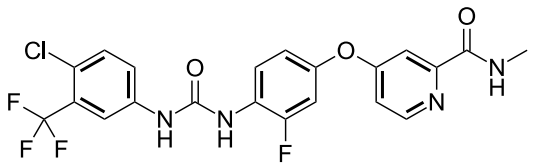

*Figure 7: **Structural formula of Regorafenib** – From Regorafenib, Wikipedia. Accessed from https://en.wikipedia.org/w/index.php?title=Regorafenib&oldid=952660384 [51].*

Regorafenib is a small-molecular multi-kinase inhibitor that only differs from sorafenib by one additional fluor atom (see Figures 5 and 7). Therefore, its mechanism of action and toxicity profile are similar to sorafenib's [52]. In comparison to sorafenib, regorafenib displays a broader span of target points, e.g. TIE2 and RET, and a higher potency towards VEGFR2 and

KIT(see Figure 4) [36]. The clinical dosage is 160mg p.o. once daily for 3 weeks in a 4-week cycle [7].

After an uncontrolled, open-label, phase II study showed regorafenib to be effective as a second-line therapy against advanced HCC after previous sorafenib treatment with a tolerable toxicity profile [53], the randomized, double -blind, phase 3 RESORCE trial was conducted. 567 patients which had previously been treated with sorafenib, sustained it but exhibited progression of their disease, were separated into two cohorts, one treated with regorafenib plus BSC, and the other solely BSC. The median OS in the regorafenib plus BSC group was 10.6 months vs 7.8 months in the BSC group. The most common AEs under regorafenib treatment were HFSR (52%), diarrhoea (33%), fatigue (29%) and hypertension (23%) [7, 54].

Following the RESORCE trial, regorafenib was approved in 2017 as a second-line therapy for advanced HCC in patients that have been treated with and tolerated sorafenib but exhibited disease progression [2].

2.4.3.3.2 Cabozantinib

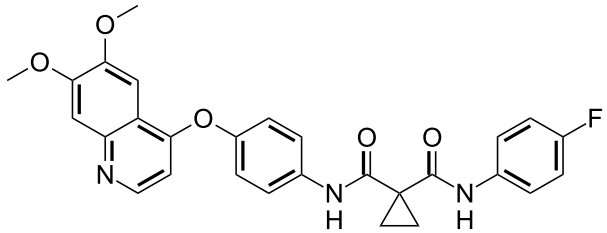

Figure 8: ***Structural formula of Cabozantinib*** *– From Cabozantinib, Wikipedia. Accessed from https://en.wikipedia.org/w/index.php?title=Cabozantinib&oldid=938120161 [55].*

Cabozantinib (Figure 8) is a multi-kinase inhibitor, which was approved in 2019 for second-line therapy of advanced HCC [2]. It targets the RTKs MET and VEGFR 2 with very high affinity, as well as VEGFR1+3, RET, AXL, KIT, FLT3 and others (see Figure 4) [56]. The clinical dose of cabozantinib is 60mg p.o. daily [57].

Cabozantinib showed a significantly improved OS against placebo (10.2 vs. 8.0 months) in patients with advanced HCC, as well as an improved PFS in the phase III CELESTIAL trial. The most common AEs after cabozantinib treatment were diarrhoea (54%), decreased appetite (48%), HFSR (46%) and fatigue (45%), similarly to other TKIs [8].

2.4.3.3.3 Ramucirumab

Ramucirumab is a recombinant human IgG1 monoclonal antibody that targets VEGFR2 selectively [58]. After the failed phase 3 REACH trial, the REACH-2 trial was conducted, which compared ramucirumab to placebo in patients with advanced hepatocellular carcinoma and AFP levels ≥400ng/ml. This trial showed a statistically significant improvement in OS by 1.2

months over placebo. Therefore, ramucirumab was approved for second-line therapy in patients with AFP levels ≥400ng/ml. REACH-2 is the first and so far, only positive biomarker driven phase III study for HCC. Ramucirumab is administered 8mg/kg intravenously every two weeks [50].

2.4.3.4 Other systemic substances

The anti-PD-1-antibodies nivolumab and pembrolizumab showed encouraging results in their phase II trials as a second-line therapy against advanced HCC. Unfortunately, both phase III trials failed to meet their primary endpoint of OS/PFS [59, 60].

Other treatment options currently under investigation include the combination of a TKI and CPI, such as atezolizumab/cabozantinib (COSMIC-312) or lenvatinib/pembrolizumab (LEAP-002), the combination of two CPIs, including nivolumab plus anti-cytotoxic T-lymphocyte-associated protein (CTLA)-4-antibody ipilimumab (CheckMate9DW) or anti-PD-L1-antibody durvalumab plus anti-CTLA-4 antibody tremelimumab (HIMALAYA), or the combination of locoregional with systemic therapies [32, 49].

2.4.3.5 Biomarkers and What drug to use?

If there is an indication for treating a patient with an advanced HCC with a TKI, there is no algorithm on which TKI to use in an individual to secure the best possible survival benefit. The identification of biomarkers for a specific TKI would be helpful for clinical decision making. Various markers, such as AFP, cMET, MEK, mTOR, transforming growth factor (TGF)-β, FGFR, Gylpican-3 and PD-1, have been analysed in different studies, but so far, no significant biomarker for prediction of response has been found for either of the four TKIs approved for clinical use in advanced HCCs [61, 62].

Lenvatinib and sorafenib both display a similar effect, are administered orally at the same frequency, and are tolerated equally well. So how do we decide which one of them to use in the first-, or second-line after previous CPI-treatment? Sorafenib leads to HFSR more often, so patients who work with their hands may rather receive lenvatinib. Lenvatinib induces a higher risk of developing hypertension, so patients with poorly controlled high blood pressure should rather be administered sorafenib. Another advantage of sorafenib, is that all second-line drugs have been tested after previous sorafenib but not lenvatinib treatment [36].

When choosing a second-line therapy there are also some considerations to be made. Firstly, ramucirumab and PD-(L)1 inhibitors are all administered intravenously while cabozantinib and regorafenib are administered orally and can be easily taken from home without a physician's assistance. Secondly, life threatening AEs occur more often after TKI- than ramucirumab- or CPI-intake. Furthermore, there are some clear recommendations in the guidelines as well,

e.g. ramucirumab should be considered if the AFP level is ≥400ng/ml and regorafenib can only be administered if sorafenib was tolerated well beforehand [36].

2.4.4 BCLC D - End-Stage-HCC

BCLC D is classified as patients with an end-stage HCC, very-poor performance status (ECOG 3-4) and poor liver function (Child-Pugh C). It is associated with a bad survival outcome of only 3-4 months. At this stage of the disease, palliative support, such as pain management, psychological support and help with nutrition is indicated and should be offered to every patient [2].

Treatment with sorafenib, regorafenib or CPIs, by themselves or in combination with TACE, can be administered in a palliative setting. Furthermore, local treatment of tumours or metastases can be indicated to reduce tumour burden and symptoms and increase quality of life [63].

2.5 Molecular classification of hepatocellular carcinoma

To categorize HCC further, various classification systems were introduced over the last 20 years, considering clinical, molecular, and transcriptomic factors. It is their aim to find sub-groups that will stratify the target populations in phase III trials, as well as possibly identify clinical features that function as biomarkers for a specific drug [64].

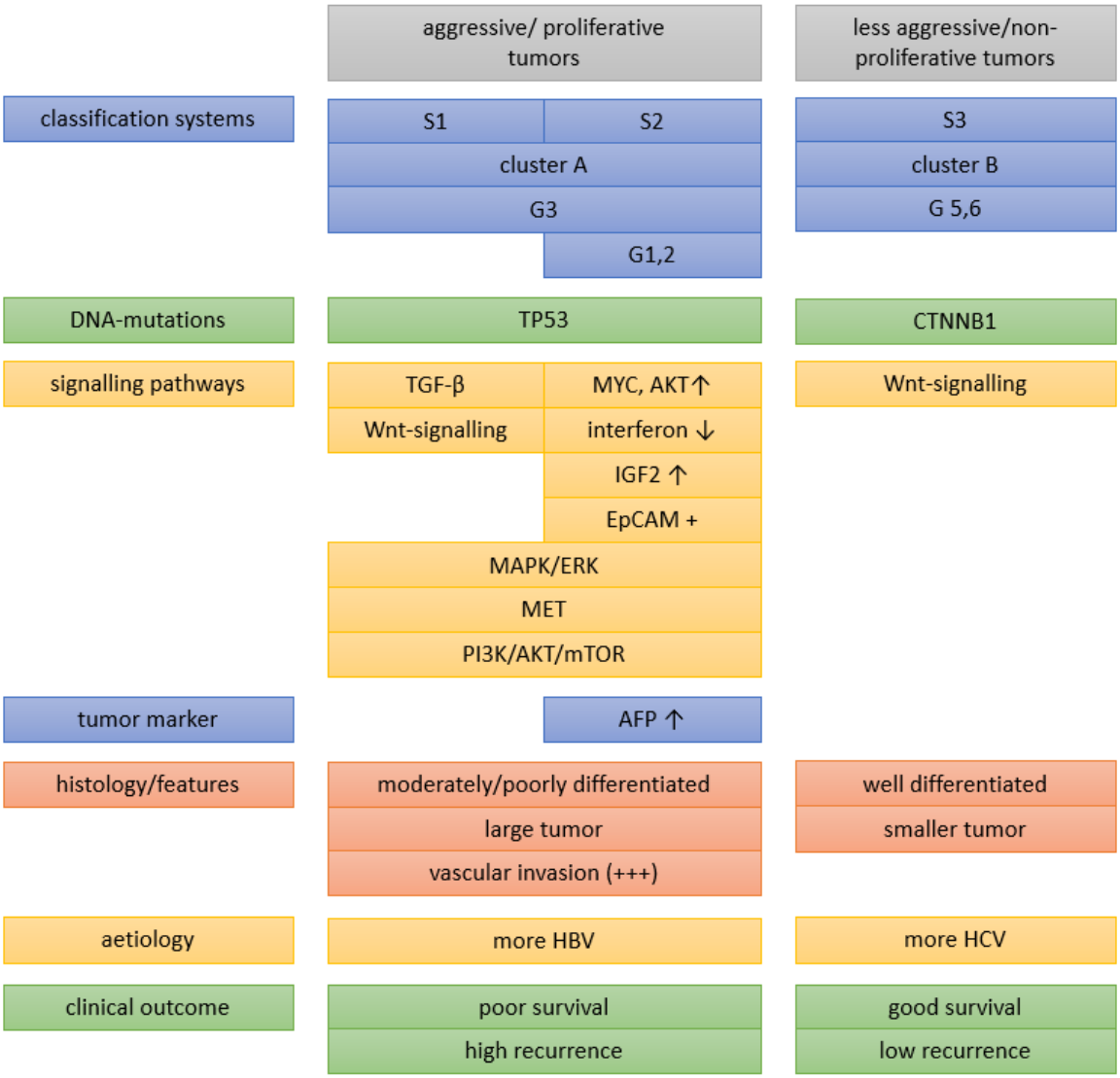

*Figure 9: **Molecular classifications of HCC.** This figure shows three different classification systems for HCC: S1-3 by Hoshida et al. [65], Cluster A/B by Lee at al. [66] and G1-6 by Boyault et al. [67]. Tumours can be clustered into aggressive/proliferative and less aggressive/non-proliferative. Abbreviations: AFP, alpha fetoprotein; AKT, protein kinase B; CTNNB1, catenin-beta 1; EpCAM, epithelial call adhesion molecule; HBV, hepatitis B virus, HCV, hepatitis C virus; IGF2, insulin-like growth factor; MAPK (=ERK), mitogen-activated protein kinases or extracellular signal-regulated kinases; mTOR, mammalian target of raptomycin; PI3K, phosphoinositide 3-kinases; TGF-β, transforming growth factor; TP53, tumour suppressor gene 53. Modified and recreated from Goossens et al. [64] and Zucman-Rossi et al. [68].*

Figure 9 shows three examples of classification systems, that are either referred to in this work or have established themselves as clinically relevant in the past.

For example, Hoshida et al. proposed the classification of HCC in three subclasses S1-S3 in 2009, based on clinical parameters and different molecular target points. S1 tumours show

Wnt signaling activation through high expression of TGF-β. Beta-catenin mutations are not seen in this subtype. Thus, inhibiting TGF-β or the Wnt signaling pathway in general could be approached when treating tumours belonging to the S1 group. S2 tumours have a high expression of both MYC and AKT, resulting in a strong activation of the PI3K/AKT/mTOR pathway. Therefore, drugs targeting this pathway should be considered as therapeutical options in tumours classified as S2. Furthermore, an elevation of serum AFP levels and insulin growth factor (IGF)-2, decreased INF levels and positive epithelial cell adhesion molecule (EpCAM) can be seen in tumours belonging to the S2 group. Both S1 and S2 are moderately to poorly differentiated, include rather large tumours, show poor survival and a high proliferation rate. In comparison, S3 tumours are smaller, well differentiated and display a good survival outcome. S3 tumours show a retained hepatocyte-like phenotype and a frequently observed mutation in the catenin beta (CTNNB)1 gene [65].

Lee et al. introduced cluster A and B, which divide HCCs by their clinical outcomes and predict OS. Cluster A has poor survival (OS 30.3 ±8.0 months) compared to cluster B (OS 83.7 ± 10.3 months), higher percentages of elevated AFP serum levels and Edmondson Grad III, faster tumour progression and a higher expression of typical cell proliferation, cell cycle markers, and regulators [66].

Lastly, Boyault et al. developed a classification by analysing clinical, genetic, and transcriptomic characteristics of 57 HCCs, and divided them accordingly into six groups, G1-G6. G1 are young patients, frequently of African heritage, with HBV-induced HCC with low copy number variations and high serum AFP- and IGF-2 levels. G2 are patients with HBV-induced HCC with high copy number variations, frequent local and vascular invasion, and mutations in TP53. G3 are patients with TP53 mutations, as well as overexpression of genes controlling the cell cycle. G4 are a heterogenous subgroup with TCF1 or PIK3CA mutations. G5 and G6 show an activation of the Wnt pathway through β-catenin and CTNNB1 mutations [67].

3. AIM OF THIS STUDY

The outcome of patients diagnosed with advanced HCC is poor and a curative treatment is not an option at this stage of the disease. It is the treatment's sole purpose to lead to a prolongment of life. There are different systemic therapies approved for clinical use for advanced HCC: CPI-combination atezolizumab plus bevacizumab, the four TKIs, sorafenib, lenvatinib, regorafenib and cabozantinib, and the anti-VEGFR2 antibody ramucirumab. Whether one drug is approved for first- or second-line is merely based on empirical data. Problematically, there are no biomarkers, neither clinical nor transcriptomic, to help us decide which TKI to use in the first- or second-line to give an individual patient the best possible outcome [3].

In this study, we set out to directly compare the four TKIs, sorafenib, lenvatinib, regorafenib and cabozantinib, to each other. The intent is to show that each TKI will induce a different response in various individuals and one individual will show a different reaction to the four TKIs. If we can support this assumption, this will further validate the need of a biomarker guided treatment algorithm. Additionally, we asked ourselves if a drug, approved for second-line treatment for advanced HCC might be more effective than a drug approved for the first-line and should therefore be evaluated as a first-line therapy option itself.

To achieve this goal, we conducted an *in vitro* viability assay with the four TKIs already approved for clinical use in patients with advanced HCC utilizing nine hepatoma cell lines with different origins, clinical markers, and transcriptomic features. In the second part of our study, we conducted a cell cycle analysis on two cell lines to determine how the four drugs are interfering in the cell lines' cell cycle.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Compounds

Cabozantinib – XL184	AdooQ Biosience LLC, Irvine, CA, USA
Lenvatinib – E7080	AdooQ Biosience LLC, Irvine, CA, USA
Regorafenib – BAY73-4506	AdooQ Biosience LLC, Irvine, CA, USA
Sorafenib – BAY43-9006	AdooQ Biosience LLC, Irvine, CA, USA

4.1.2 Cell lines

Hep 3B	Well-differentiated HCC cell line from an 8-year-old Black male from USA Established by Aden DP et al. in 1979 [69] Obtained from American Type Culture Collection (ATCC)
Hep G2	Hepatoblastoma-cell line from a 15-year-old Caucasian male from Argentina Originally wrongly described as a well-differentiated HCC Established by Aden DP et al. in 1979 [69] Obtained from ATCC
HLE	Undifferentiated hepatoma cell line from a 68-year-old Asian male Established by Doi I et al. in 1975 [70] Kindly provided by Dr. rer. nat. Steven Dooley (University of Mannheim, Germany)
HLF	Undifferentiated hepatoma cell line from a 68-year-old Asian male HLE and HLF were derived from the same patient, with HLE being typically epithelial-like and HLF fibroblast-like but originating from hepatoma cells Established by Doi I et al. in 1975 [70] Kindly provided by Dr. rer. nat. Steven Dooley (University of Mannheim, Germany)
HuH1	HCC cell line from a 53-year-old Asian male from Japan Established by Huh N, Utakoji T in 1981 [71] Kindly provided by Dr. rer. nat. Steven Dooley (University of Mannheim, Germany)
HuH7	Well-differentiated HCC cell line from a 57-year-old Asian male from Japan Established by Nakabayashi H et al. in 1982 [72] Obtained from ATCC
PLC-PRF5	HCC cell line from a 24-year-old Black male from Mozambique Established by Alexander JJ et al. in 1976 [73] Obtained from ATCC

Snu398	Anaplastic HCC cell line from a 42-year-old Asian male from Korea The patient was pre-treated with TAE with doxorubicin + mitomycin-C Established by Park JG et al. in 1995 [74] Kindly provided by Dr. rer. nat. Steven Dooley (University of Mannheim, Germany)
Snu475	Poorly-differentiated HCC cell line from a 43-year-old Asian male from Korea The patient received no prior treatment Established by Park JG et al. in 1995 [74] Kindly provided by Dr. rer. nat. Steven Dooley (University of Mannheim, Germany)

4.1.3 Cell culture

6- and 96-well plates	Eppendorf AG, Hamburg, Germany
10cm dishes	Eppendorf AG, Hamburg, Germany
Cell culture flasks 15ml	Corning B.V. Life Sciences, Amsterdam, Netherlands
Cell culture flasks 50ml	Sarstedt AG & Co. KG, Nümbrecht, Germany
CRYO.S^{TM} tubes	TPP Techno Plastic Products AG, Trasadingen, Swizerland
Cyprofloxacin 400mg/200ml	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
Dimethylsulfoxid (DMSO)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich, St. Louis, MO, USA
DMEM/Nutrient Mixture F-12 Ham (DMEM/F-12)	Sigma-Aldrich, St. Louis, MO, USA
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich, St. Louis, MO, USA
Filtrated Bovine Serum (FBS)	PAN-Biotech GmbH, Aidenbach, Germany
Glass pasteur pipettes	Brand GmbH & Co. KG, Wertheim, Germany
L-Glutamine 200mM	Sigma-Aldrich, St. Louis, MO, USA
Neubauer counting chamber	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Penicillin-Streptomycin (P/S)	Sigma-Aldrich, St. Louis, MO, USA
Pipette filler Pipetus®	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany

Pipettes Pipet-Lite XLS - various sizes	Mettler-Toledo, Columbus, OH, USA
Pipette tip 20µl, 200µl, 2000µl	Mettler Toledo, Columbus, OH, USA
RPMI-1640 medium	Sigma-Aldrich, St. Louis, MO, USA
Safe Seal tubes 0.5ml, 1.5ml	Sarstedt AG & Co. KG, Nümbrecht, Germany
Serologic pipettes 5ml, 10ml	Sarstedt AG & Co. KG, Nümbrecht, Germany
Serological pipettes 25ml	TPP Techno Plastic Products AG, Trasadingen, Swizerland
Trypsin/EDTA solution 10x	Sigma-Aldrich, St. Louis, MO, USA
Tryptan Blue solution 0.4%	Sigma-Aldrich, St. Louis, MO, USA

4.1.4 FACS reagents

BD™ Extended Flow Cell Clean Solution	Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA
Cleaning Concentrate Solution	Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA
Natriumcitrat	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Natriumhypochloridlösung	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Propidium iodide	Sigma-Aldrich, St. Louis, MO, USA
SYBR™ Green/Nucleic Acid Gel Stain	Lonza, Rockland, ME, USA
Triton X 100	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

4.1.5 Instruments

BD Accuri™ C6 Plus Flow Cytometer	Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA
Centrifuge Hettich Rotina 380R	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Centrifuge Hettich Rotina 420R	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Cytofluor® Multi-Well Plate Reader Series 4000	PerSeptive Biosystems, Inc, Framingham, MA, USA
GFL 1004 Water Bath Type 1004	Gesellschaft für Labortechnik m.b.H. & Co., Burgwedel, Germany
Heracell CO_2 incubator	Heraeus, Hanau, Germany

HERAsafe® Safety cabinet HS	Heraeus, Hanau, Germany
IIX50 Inverse Microscope	Olympus K.K., Shinjuku, Tokio, Japan

4.1.6 Software

BD Accuri C6 software	Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA
GR calculator	HMS LINCS Center, Harvard Medical School, Boston, MA, USA
GraphPad Prism 8	GraphPad Software Inc., San Diego, CA, USA
IBM SPSS Statistics	SPSS Inc., IBM, Armonk, NY, USA
Microsoft office 365	Microsoft, Redmond, WA, USA

4.2 Methods

4.2.1 Cell culture

The cell lines Hep3B, HepG2, HuH7 and PLC-PRF5 were obtained from ATCC. The cell lines HLE, HLF, HuH1, Snu398 and Snu475 were kindly provided by Dr. rer. nat. Steven Dooley of the University of Mannheim, Germany. All cell lines have been authenticated by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

HepG2, HLE, HLF, HuH1, HuH7 and PLC-PRF5 were cultivated in DMEM, Hep3B in DMEM/F12, supplemented with 200mM L-glutamine, and Snu398 and Snu475 in RPMI-1640 medium. All media were supplemented with 10% Filtrated Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S). Cells were constantly maintained at 37°C and 5% CO_2.

The medium was changed every two to three days and the cells were split when approximately 70-90% confluency was reached, depending on the cell line, but at least once a week. When splitted, the old medium was removed, 0.1% Trypsin/EDTA-solution, diluted in Dulbecco's Phosphate Buffered Saline (PBS), was added, and after resting for 3-8 min at 37°C and 5% CO_2, medium was added to the Trypsin/EDTA-cell-compound and the cells were splitted in a 1:2-10 ratio, depending on the rapidity of cell growth.

When freezing cells, the cell-medium-suspension after the splitting process was centrifuged at 1250 rpm and 4°C for 10min. Afterwards, the medium was removed and the remaining pellet was resuspended in a medium solution containing 5% DMSO, which was then put into a cryo-tube and gradually frozen. For thawing, the cells were put into a waterbath at 37°C for no longer than 2min, transferred into 10ml of medium and centrifuged at 1250 rpm and 20°C for 8min. Then, the medium was removed, the remaining pellet was resuspended in medium and put in a 10cm dish. Each cell line was splitted at least twice after the thrawing process before being used for an experiment.

All cell lines were tested for mycoplasma infection using polymerase chain reaction. Initially, five out of the nine cell lines were tested positive for mycoplasma. Those cell lines were either treated with 10µg/ml ciprofloxacin for 21 days or a new batch of the same cell line was thawed and tested again. Afterwards all cell lines were tested negative for mycoplasma infection. All experiments, that have been conducted before the mycoplasma testing with cell lines that have tested positive for mycoplasma infection, were repeated and if a significant deviation was detected, they were excluded from further analysis.

4.2.2 Cell viability assay

4.2.2.1 Drug treatment

Cabozantinib, lenvatinib, regorafenib and sorafenib were dissolved in 100% DMSO at a stock concentration of 100µM and stored at -80°C until used for an experiment. For each experiment, five 96-well plates were seeded with 800 to 3000 cells per well, depending on the rapidity of a cell line's growth, and then left to attach for approximately 24 hours. Four out of the five 96-well plates were then treated with seven different concentrations of each drug, ranging from 0.03µM to 20µM in a 1:2 to 1:3 serial dilution. The fifth plate did not undergo drug treatment. As a control a DMSO-medium solution was used, with the DMSO concentration being equivalent to the amount of DMSO in the highest drug concentration. Each experiment was done in triplicates with at least three individual biological replicates per agent.

4.2.2.2 Cell count

The cell count for the cells previously treated with a drug was estimated after six days of drug incubation, and for the untreated cells four hours after the seeding process. To estimate the cell count, the old medium was removed, the cells were washed with PBS twice, underwent osmotic lysis by adding 100µl of double distilled water and were incubated at 37°C and 5% CO_2 for 45 to 60 min. 100µl of 0.2% SYBR green diluted in double distilled water was then added to each well and fluorescence was measured, using the Cytofluor Series 4000 [75]. The proliferation index was then calculated as the ratio of the treated cells at concentration c to the DMSO control samples. Afterwards, the IC_{50} and E_{max} values for each cell line and drug were calculated. The IC_{50} is the half maximal inhibitory concentration, which shows the concentration of drug at which the cell count is 50% of the control, and therefore a metric for potency. The E_{max} represents the maximal efficacy, which stands for the cell count at the highest drug concentration and is therefore a metric for efficacy.

4.2.2.3 Growth rate metrics

Hafner et al. stated that "if cells undergo different numbers of divisions during the course of an assay due to natural differences in proliferation rate (PR), variation in growth conditions, or changes in the duration of an experiment, IC_{50} and E_{max} […] values will vary dramatically, independent of any changes in underlying biology" [76].

The growth rate inhibition (GR) method, established by Hafner et al. in 2016, aspires to minimize the confounding effects of the varying division rates on drug response assays by factoring in the division rate itself in their calculations. The normalized growth rate inhibition in the presence of drug at concentration c was calculated as follows:

$$GR(c) = 2^{\frac{log_2(x(c)/x_0)}{log_2(x_{ctrl}/x_0)}} - 1$$

with x(c) being the mean of triplicates following drug treatment at concentration c at day 7, x_{ctrl} being the mean of triplicates of DMSO treated control wells from the same plate at day 7 and x_0 being the mean of triplicates from untreated samples from the time of treatment at day 1. Typically, the GR value lies between -1 and +1, whereas a positive value (0 to +1) correlates to partial growth inhibition, GR equals 0 stands for complete cytostasis, i.e., no increase or decrease in cell number, and negative values (0 to -1) stand for cytotoxicity [76, 77].

Afterwards the GR values were fitted to a sigmoid curve and the GR_{50} and GR_{max} values were calculated. The GR_{max} value represents the GR value at highest concentration of the drug. It is a metric for drug efficacy and typically lies between -1 and +1. The algebraic sign corresponds directly to the response phenotype: cytotoxicity, cytostasis or partial growth inhibition, as previously described. The GR_{50} value represents the concentration of drug at which GR(c) = 0.5 and is a metric for drug potency [76, 77].

Even though, IC_{50} and E_{max} were also calculated and graphed, only the GR_{50} and GR_{max} values were used for further analysis.

4.2.3 Cell cycle analysis

The cell cycle analysis was conducted on two cell lines with all four TKIs with the assay's endpoint being 24, 48 or 72 hours after drug incubation. Each well of 6-well-plates was seeded with 70,000-120,000 cells of HuH7 or 40,000-80,000 cells of HLE, depending on the assay's endpoint. The aspired cells' density was determined in a previously conducted trial experiment. After seeding, the cells were left to attach for roughly 24 hours at 37°C and 5% CO_2.

After 24 hours, the medium was aspirated and a drug-medium-solution, containing one of the four TKIs, was added on the cells. On each well-plate, two wells were assigned the control group without any drug stimulation, two concentration 1 and two concentration 2. The concentrations were chosen after careful evaluation of the previously conducted cell viability assays and are as followed: 1.25µM and 2.5µM for sorafenib and regorafenib, 2.5µM and 5µM for lenvatinib, and 5µM and 10µM for cabozantinib. Afterwards, the cells were incubated at 37°C and 5% CO_2.

After 24, 48 or 72 hours the endpoint of the assays was reached. From this point onwards, all work was done on ice, to slow down the metabolism of the cells and prevent them from going into cell death. The supernatant medium was aspirated, the wells washed twice with 500µl of PBS and all media was collected in a 15ml Falcon tube. Then, approximately 300µl of Trypsin-EDTA-solution was added and the cells were left to incubate for 2-3 minutes at 37°C and 5%

CO_2. Medium was added to the trypsin-cell-compound, the cells were cautiously detached from the subsurface of the well plate and added to the falcon tubes. The falcon tubes were then centrifuged for 10 minutes at 4°C and 1250 rpm twice, with intermediate PBS washing. After the second centrifugation, the remaining cell pellet was resuspended in 300µl of Nicoletti solution and put into 1ml-Eppendorfer tubes, which were stored at 4°C for at least three hours. The Nicoletti solution had been prepared beforehand with 0.1% Natriumcitratdehydrate, 0.1% Tritan-x-100, 50µg/ml Propidiumiodid and double-distilled-water and stored at 4°C until usage.

Lastly, fluorescence activated cell sorting was performed on the propidium iodide-stained cells using the Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were separated in the G1-, S- and G2-phases, as well as apoptotic cells, which was defined as sub-G1.

4.2.4 Graphics

All graphics and figures were generated using GraphPad Prism 8 or PowerPoint.

4.2.5 Statistical analysis

Data were analysed by IBM SPSS Statistics version 27. Results are shown as mean +/- standard deviation (SD) of at least three individual experiments.

To assess the statistical difference of our results, a one-way analysis of variance (ANOVA) or the more robust Welch-ANOVA, followed by a post-hoc test was carried out for the results of the drug assay as well as the fluorescence activated cell sorting (FACS) analysis. First it was evaluated if the requirements for using ANOVA-analysis were met. Thus, we checked that the samples were independent, the dependent variable was interval scaled and the independent variable nominal scaled. Then the Shapiro-Wilk test was used, showing if all cell lines were normally distributed at a specific concentration and drug (normally distributed if $p>0.05$). Outliers were identified by looking at a box plot. Slight outliers, thus greater than 1.5 but less than 3 times of interquartile range, were ignored. Extreme outliers, thus greater than 3 times of interquartile range, were inspected again and taken out of the analysed data if needed. Using Levene's test, the homogeneity of variances was assessed, showing equal variances if $p<0.001$. Only if all these requirements were met, a one-way ANOVA test was conducted. If all requirements were met but equal variances could not be shown, the modified, more robust Welch-ANOVA was conducted. Both the ANOVA- and the Welch-ANOVA test showed a statistically significant difference between at least two cell lines if $p<0.05$. To determine which cell lines, show a statistically significant difference in effect to one drug, a post-hoc analysis was conducted. After ANOVA, Turkey-post-hoc-analysis was carried out. If equal variances could not be shown and the more robust Welch-ANOVA was used, a Games-Howell analysis

was conducted. If $p<0.05$ between two specific cell lines, these two cell lines showed a statistically significant difference in response [78].

Additionally, when comparing our GR_{50} values with our IC_{50} values, our GR_{max} values, Caruso et al.'s GI_{50} values [10] and Qiu et al.'s IC_{50} values [9], we calculated the correlation coefficients (r) using IBM SPSS statistics, to determine whether there is a correlation between two factors and its strength. Firstly, we checked if the correlation between two factors was linear using a scatter plot diagram as well as a fit of the curve analysis, checking for linear, cubic, quadratic, logarithmic or invers correlations. If two factors correlated linearly, we calculated Pearson's correlation coefficient. If any other type of correlation existed, we calculated the more robust Spearman's Rho correlation coefficient, because in these cases the correlation coefficient according to Pearson would underestimate the strength of correlation. The strength of correlation was then interpreted according to Cohen: $r < 0.1$ shows no, $0.1 \leq r < 0.3$ a small, $0.3 \leq r < 0.5$ a medium and $r \geq 0.5$ a strong correlation between two factors [79]. P-value < 0.05 showed that the corresponding correlation was statistically significant [80].

5. RESULTS

5.1 Cell line characteristics

Nine hepatoma cell lines were used in this study (see Figure 10), one of which was derived from a hepatoblastoma (11.1%) and the remaining eight from hepatocellular carcinomas (88.9%). All cell lines were derived from male patients. Five out of nine cell lines are HBV surface antigen (HBs-Ag) positive (55.6%) and five out of nine show elevated AFP levels (55.6%). None of the cell lines were tested positive for an HCV infection, although only one has been tested negative for an HCV infection (11.1%). Two cell lines were derived from Black patients (22.2%), one from a Caucasian patient (11.1%) and six from Asian patients (66.7%). Three patients were in the age cohort 0-25 years (33.3%) and six were over 25 years old (66.6%). Five cell lines are well to moderately differentiated (G1-2, 55.6%) and four poorly differentiated to undifferentiated (G3-4, 44.4%). Six cell lines can be classified as S2 tumours (66.7%) and three as S1 tumours (33.3%), according to Hoshida et al. [65].

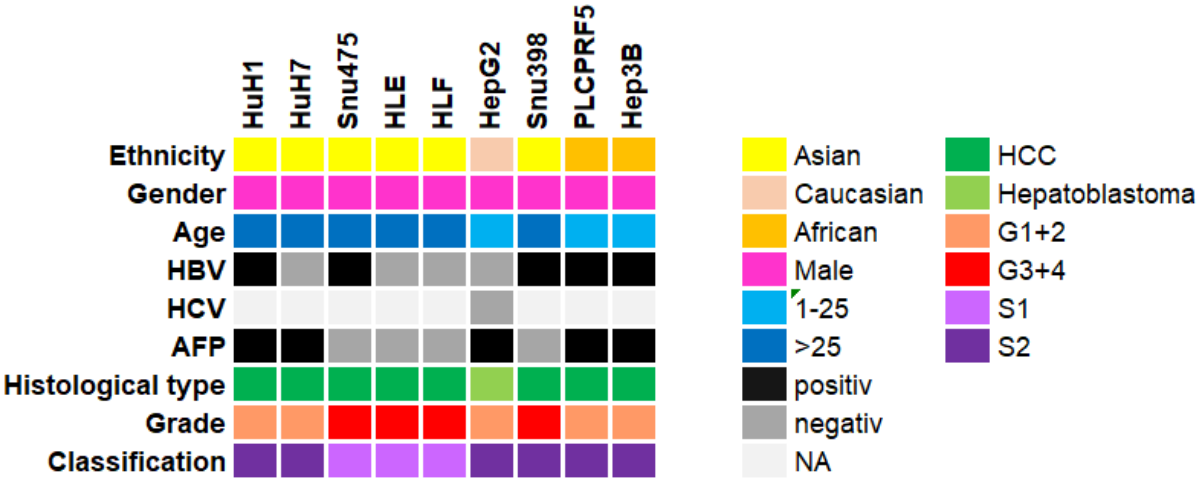

Figure 10: ***Clinical and histological features of nine hepatoma cell lines.*** *This figure displays some typical features of the nine hepatoma cell lines that have been investigated in this study, including their ethnicity, gender, age, HBV- and HCV-infection status, AFP level, histological type, grading and classification according to Hoshhida et al. [65]. Abbreviations: AFP, alpha feto-protein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NA, non-applicable. Information on cell lines [69-74]. Modified and recreated from Hirschfield et al. [81], Qui et al. [9] and Caruso et al. [10].*

5.2 Considerations prior to starting the viability assays

5.2.1 Evaluation of TKI dose finding

Before starting our cell viability assays, the question arose what concentration to use for each individual drug. To determine the suitable range of concentrations, we looked at the steady state plasma concentration (SS) of each drug after multiple doses, examined various pharmacokinetic studies to assess what ranges have been used in similar studies and lastly, conducted trial experiments with two different concentration ranges, starting at 20µM in a 1:2 serial dilution and 100µM in a 1:10 serial dilution.

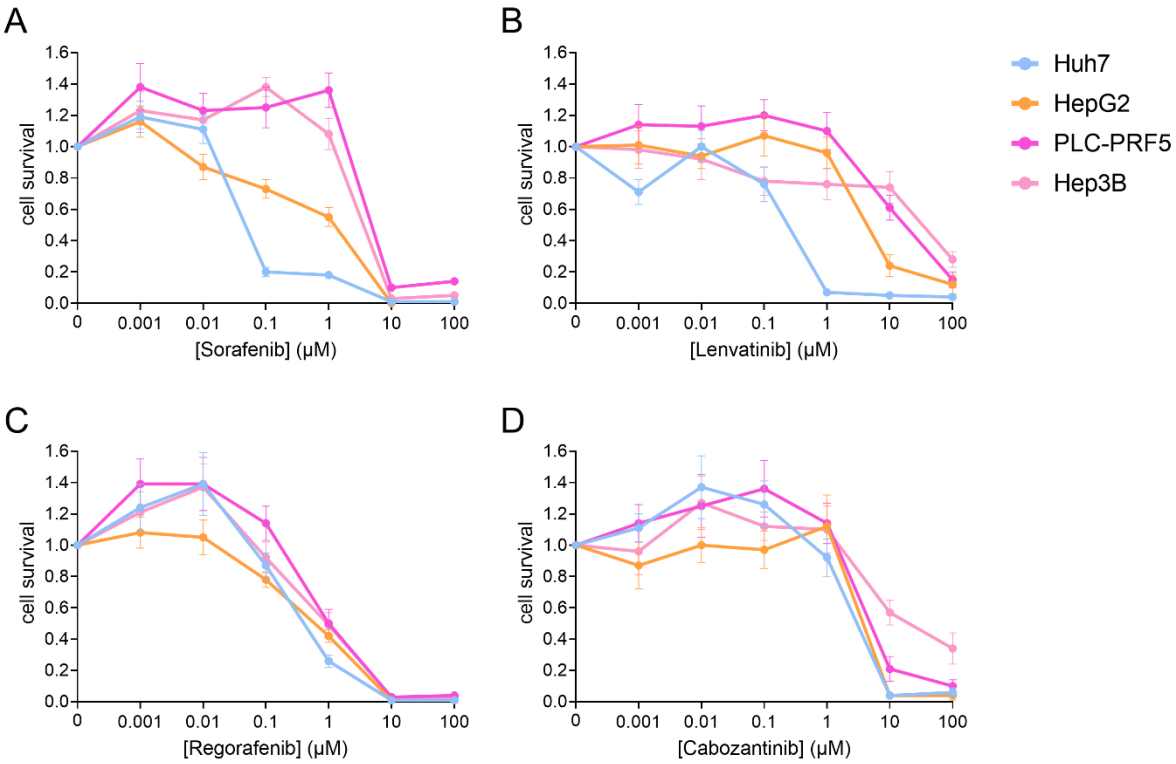

Figure 11: ***Trial experiments with a maximum concentration of 100µM in a 1:10 serial dilution for A sorafenib, B lenvatinib, C regorafenib and D cabozantinib.*** *This figure shows the results of one of the trial experiments that has been conducted to identify the fitting concentration ranges for each drug. To save resources, only four out of the nine cell lines, that have been known to show a broad variability in response to the different drugs in previous experiments, have been chosen to have the trial assays performed on them. Results are presented as the mean values plus standard deviation (SD) of two individually conducted experiments.*

In retrospect, it is apparent that a maximum concentration of 100µM is too high, in the instance of the sorafenib and regorafenib experiments since few cells were viable after being treated with a concentration of 10µM (see Figure 11A + C). The concentration at which 50% of the cells are no longer viable, and therefore the concentration range of interest, lies somewhere between 0.1µM and 10µM. Given that this observation could be seen for all four drugs, a smaller serial dilution was to be aspired for all drugs. To find the optimal concentration range, two more trial experiments were conducted, one with a maximum concentration of 5µM and

one with a maximum concentration of 10µM, both with a 1:2 serial dilution. In a previous paper, Carr et al. used a concentration range for regorafenib of 1µM to 10µM for 3 to 7 days. [82], Liu et al. used a sorafenib concentration range of 0.01µM to 10µM for a three day incubation under drug stimulation [83] and Ye et al. used a concentration range for sorafenib of 0.31µM to 20µM for a duration of six days [84]. After much consideration, we decided to use a maximum concentration of 5µM in a serial dilution of 1:2 for both sorafenib and regorafenib, even though the steady state concentration of regorafenib after a dosage of 160mg p.o. daily was roughly 8.08µM [85] and the steady state concentration of sorafenib after 400mg bid p.o. over a duration of 28 days was 11.55 µM [86] and therefore not included in our decided upon concentration ranges.

The maximum plasma concentration of lenvatinib after administering 12mg p.o. daily for four weeks was 0.78µM [87]. Utilizing lenvatinib, Rodríguez-Hernández et al. treated their cell cultures for one day at drug concentrations ranging from 0.1µM to 100µM [88], while Sasaki et al. decided upon a concentration range of 1µM to 20µM over two days [89]. Based on our trial experiment with lenvatinib (see Figure 11B), we concluded that the maximum concentration of 100µM, as well as the serial dilution of 1:10 were unnecessarily high, leading us to conduct all following experiments with a maximum concentration of 20µM of lenvatinib in a 1:3 serial dilution.

The steady state concentration of cabozantinib after multiple doses with 140mg for 29 days was 3.27 µM [90]. Given that the therapeutical dose for patients with HCC is only 60mg p.o. once daily, the steady state concentration of interest might even be smaller. Xiang et al. conducted an experiment with a cabozantinib concentration range of 10^{-5}µM to 100µM over the duration of three days [91] and Rodríguez-Hernández with a range of 0.1µM to 100µM over the duration of one day [88]. The first trial experiment we conducted with a maximum concentration of 100µM and a 1:10 serial dilution (see Figure 11D) showed that the drug range of interest is somewhere between 0.1 and 10µM. Therefore, a second experiment with a maximum concentration of 20µM in a 1:2 serial dilution was conducted. It showed various concentration points with surviving fractions between 1.0 and 0.0 and all cell lines came near a surviving fraction of 0.0. Hence our concentration range of choice was 0.31µM to 20µM for cabozantinib, which is lower than others have used before us, but can be justified by the longer duration of the drug assay, i.e., six days.

5.2.2 DMSO control

All drugs were dissolved in DMSO to attain a 100µM concentration, according to the manufacturer's instructions. Given that DMSO has a known cytotoxic effect [92], we next determined if DMSO is partially responsible for the decreased cell survival associated with the test articles. Hence, we conducted an experiment using a DMSO-medium-compound, with various concentrations of DMSO equivalent to the concentration of DMSO in the TKI-medium solution, used for the drug assay experiments.

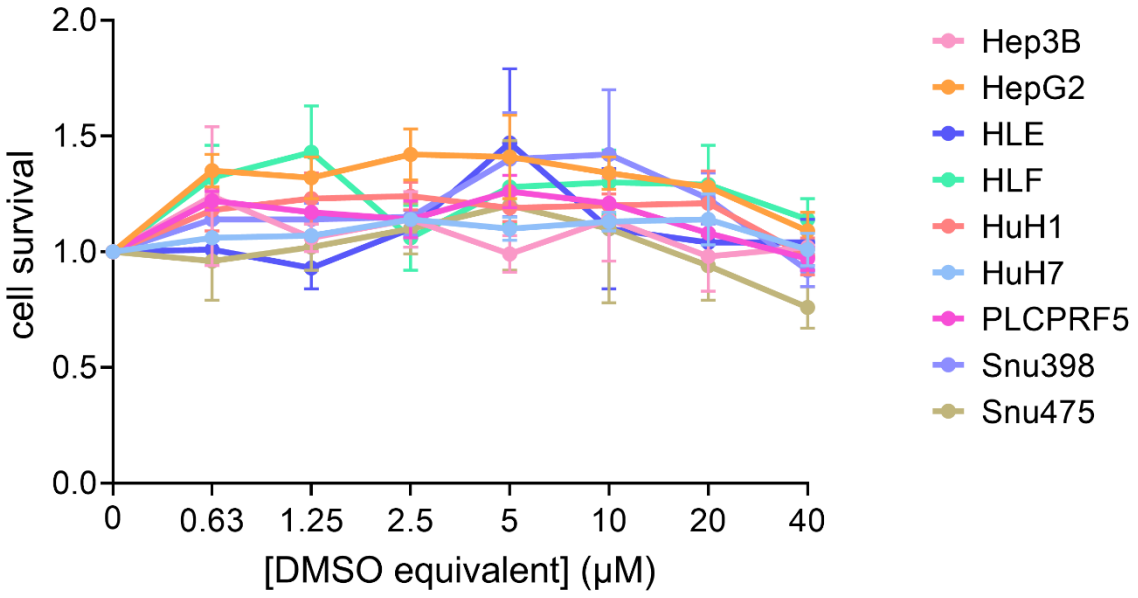

Figure 12: ***Effect of DMSO-medium-solution in various concentrations on all nine hepatoma cell lines.*** *The DMSO-medium-solutions are equivalent to the concentration of DMSO in the drug compound that has been used for the viability assays. Thus, a 20µM DMSO equivalent solution contains as much DMSO as a 20µM drug-medium-solution that has been used in the viability assays. Results are shown as the mean and SD values for at least three experiments.*

Figure 12 shows that DMSO does not have a significant cytotoxic effect in the concentrations that have been used to dissolve the tyrosine kinase inhibitors, and therefore cannot be held responsible for the decrease in cell survival after drug treatment.

5.2.3 Cell lines' proliferation rate (PR)

Figure 13 and Table 6 show the proliferation rate of each cell line over seven days, as well as per day. The overall proliferation rate is calculated as the logarithmic ratio of the cell count at day 7 to the cell count at day 0. To calculate the proliferation rate per day, the overall proliferation rate was divided by seven.

	Overall PR	SD overall PR	PR per day	SD PR per day
HuH7	7,36	0,19	1,05	0,03
HLE	6,46	0,23	0,92	0,03
Snu398	5,99	0,68	0,86	0,10
HLF	5,11	0,35	0,73	0,05
HuH1	5,14	0,40	0,73	0,06
Hep3B	4,74	0,41	0,68	0,06
PLC-PRF5	4,55	0,11	0,65	0,02
HepG2	4,13	0,68	0,59	0,10
Snu475	0,33	0,43	0,05	0,06

*Table 6: **Proliferation rate.** This table shows the proliferation rate (PR) and SD overall, that is over seven days, and per day, for each cell line. Cell lines are in order of proliferation rate (highest to lowest). Results are shown as mean and SD values for at least three experiments.*

HuH7, HLE and Snu398 are the fastest proliferating cell lines that have been used in this study (PR per day >0.8). HLF, HuH1, Hep3B, PLC-PRF5 and HepG2 are in the centre span with a PR per day between 0.8 and 0.5. Snu475 stands out as the slowest proliferating cell line, with a proliferation rate per day of 0.05, which is over eleven times slower than the next slowest proliferating cell line.

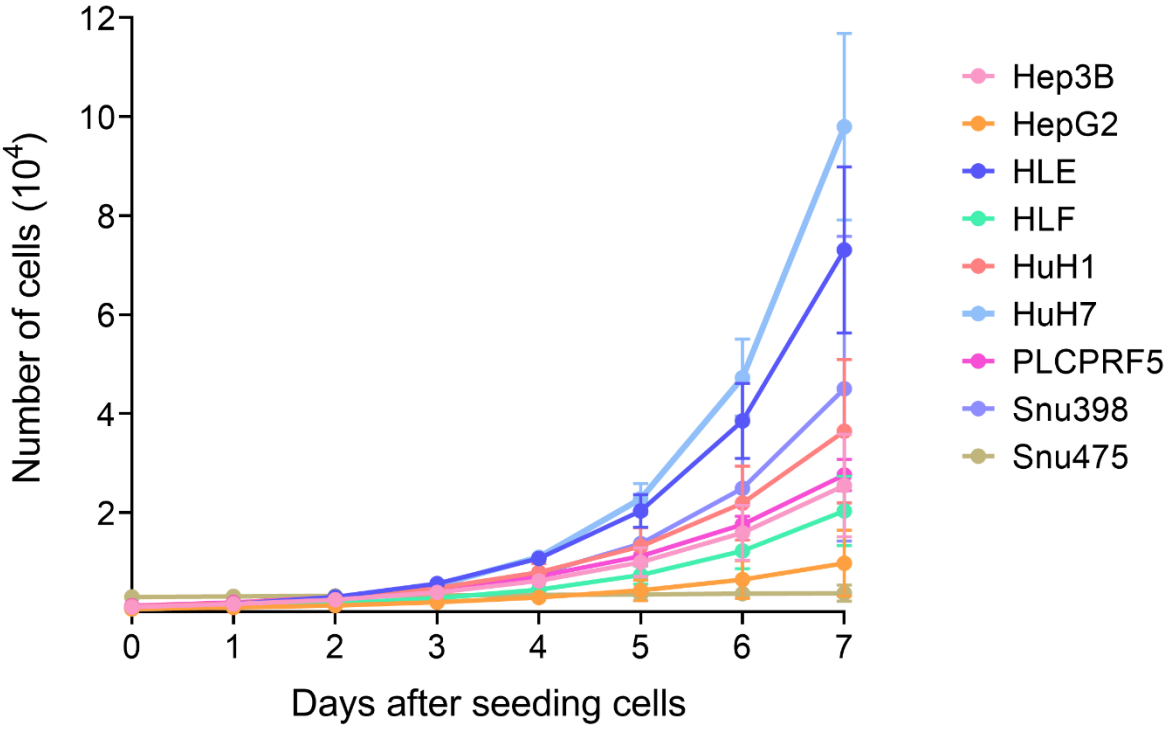

*Figure 13: **Cell lines' proliferation over seven days.** This figure represents the rapidity of each cell line's growth. We evaluated the number of cells at day 0, thus four hours after seeding, as well as day 7. Assuming an exponential cell growth, the proliferation rate per day was calculated. Results are shown as the mean and SD values for at least three experiments.*

5.3 Cell viability assay

The effect of the four multi-tyrosine kinase inhibitors, sorafenib, lenvatinib, regorafenib and cabozantinib, on nine different hepatoma cell lines has been investigated in this study. Prior to starting this project, we asked ourselves two questions: Firstly, does one drug have the same effect on different cell lines? And secondly, does one cell line react the same to different drugs?

5.3.1 According to drugs

Figures 14 A-D show the effect of the four TKIs on the nine hepatoma cell lines. As previously described, we decided on using a six-day treatment protocol to better mimic the clinical application of TKIs. Each TKI induces a decrease in cell growth in all cell lines in a dose- and time-dependent manner.

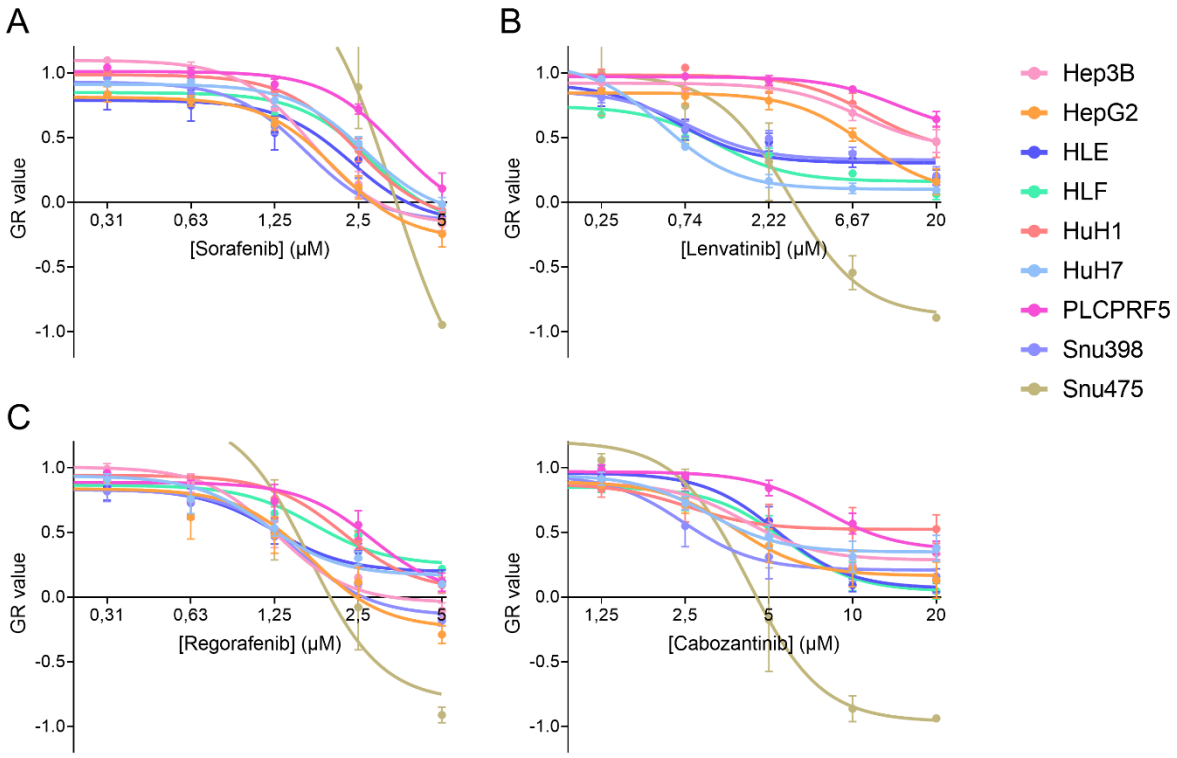

Figure 14: ***The effect of one tyrosine kinase inhibitor on all cell lines****.* ***A*** *Effect of sorafenib on all cell lines with a concentration range of 0.08µM to 5µM in a 1:2 serial dilution.* ***B*** *Effect of lenvatinib on all cell lines with a concentration range of 0.03µM to 20µM in a 1:3 serial dilution.* ***C*** *Effect of regorafenib on all cell lines with a concentration range of 0.08µM to 5µM in a 1:2 serial dilution* ***D*** *Effect of cabozantinib on all cell lines with a concentration range of 0.31µM to 20µM in a 1:2 serial dilution Results are presented as the mean and SD values for at least three experiments. P<0.05 for all drugs by ANOVA. Due to an increased proliferation of Snu475 under sorafenib and regorafenib at the lower concentrations and to better fit the representation in this graph and compare to the other cell lines, we do not show Snu475 at said lower concentrations for these two drugs. The complete dose response curve of Snu475 can be seen in figure 16 I. Adapted from Sagmeister P, Daza J, et al. Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis. J Hepatocell Carcinoma. 2022;9:595-607 [12].*

Figure 14A shows the effect of sorafenib on all nine cell lines in a concentration range of 0.08µM to 5µM in a 1:2 serial dilution. While the efficacy, represented by the GR_{max} value, and potency, represented by the GR_{50} value, of all cell lines seem to be clustered close together,

displaying a homogenous picture, there are some differences that can be detected when taking a closer look. Hep3B, HepG2, HLE and Snu398 are more sensitive to sorafenib with GR_{50} values below 2µM, while HLF, HuH1, HuH7, PLC-PRF5 and Snu475 are less sensitive to sorafenib with GR_{50} concentrations greater than 2µM. HLE and HLF, which were created from the same initial tumour, and usually react similarly to all drugs, exhibit a slight difference in potency with sorafenib: HLE having a GR_{50} concentration of 1.3µM and HLF of 2.3µM. However, in comparison to other drugs used in this experiment, the difference in potency is very little with the highest ($GR_{50,\ S\text{-}HLE}$ 1.3µM) and the lowest ($GR_{50,\ S\text{-}PLC\text{-}PRF5}$ 2.9µM) only differing by a factor of 2.2. Looking at the GR_{max} values, most cell lines display a slight cytotoxic effect at the highest concentration of sorafenib, thus negative GR_{max} values (see Figure 15A, Table 7): $GR_{max,\ S\text{-}Hep3B}$ -0.17, $GR_{max,\ S\text{-}HepG2}$ -0.25, $GR_{max,\ S\text{-}HLE}$ -0.12, $GR_{max,\ S\text{-}HLF}$ -0.08, $GR_{max,\ S\text{-}HuH1}$ -0.06, $GR_{max,\ S\text{-}HuH7}$ -0.01, $GR_{max,\ S\text{-}PLC\text{-}PRF5}$ 0.11, $GR_{max,\ S\text{-}Snu398}$ -0.16 and $GR_{max,\ S\text{-}Snu475}$ -0.95. See also [12].

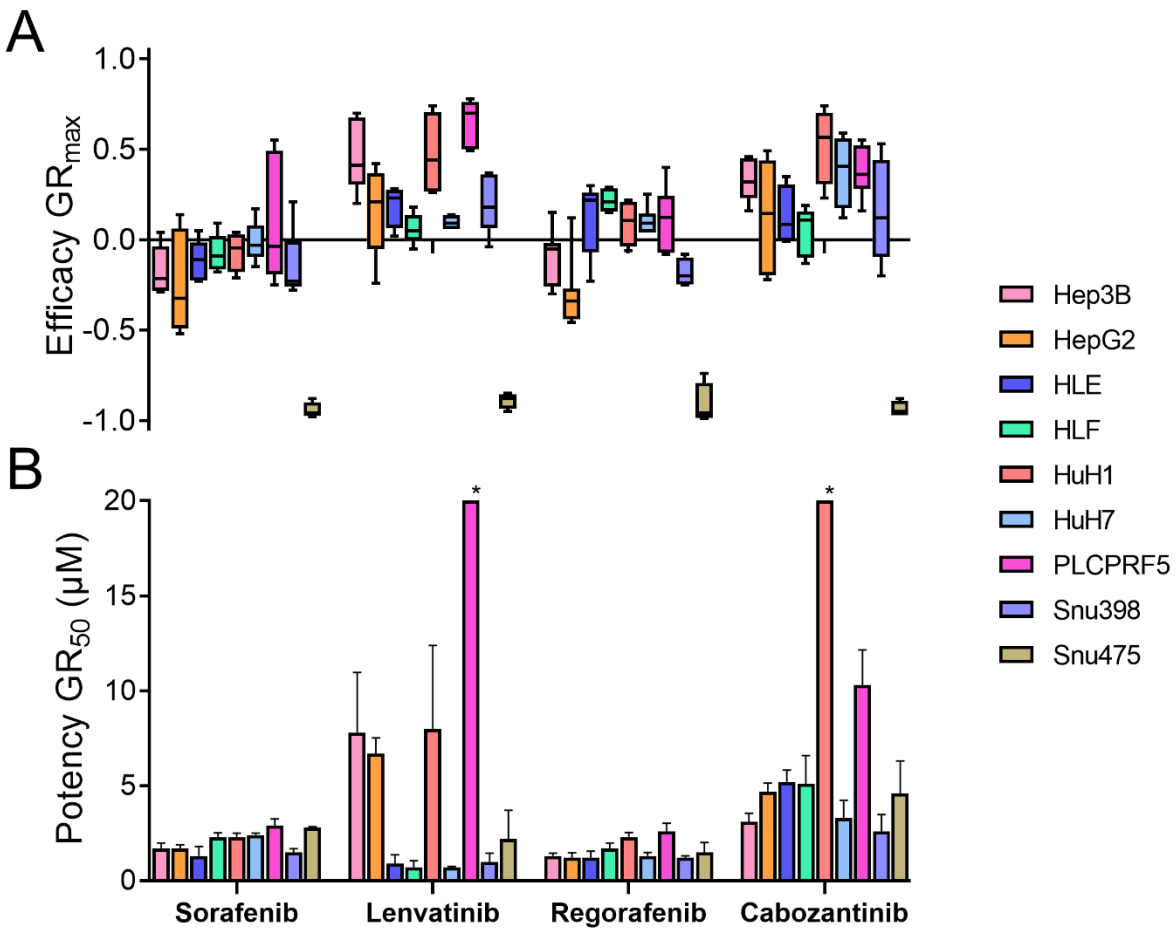

Figure 15: ***Potency and efficacy of the four TKIs for all nine hepatoma cell lines according to drugs. A*** *Efficacy (GR_{max}) of sorafenib, lenvatinib, regorafenib and cabozantinib for all nine cell lines.* ***B*** *Potency (GR_{50} in µM) of sorafenib, lenvatinib, regorafenib and cabozantinib for all nine cell lines. Results are presented as the mean and SD values for at least three experiments. P<0.05 for all drugs by ANOVA. Adapted from Sagmeister P, Daza J, et al. Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis. J Hepatocell Carcinoma. 2022;9:595-607 [12].*

The effect of lenvatinib on the nine cell lines is shown in Figure 14B, which shows the most heterogenous pattern of responsiveness out of the four drugs. When excluding Snu475, the remaining cell lines can be divided into two groups according to potency: The first group, containing HLE, HLF, HuH7 and Snu398, shows GR_{50} values between 0.7µM for HuH7 and HLF and 1.0µM for Snu398. The second group, containing Hep3B, HepG2, HuH1 and PLC-PRF5, displays GR_{50} values between 6.7µM for HepG2 and >20µM for PLC-PRF5. Hence, the GR_{50} value of the most sensitive cell lines (HuH7 and HLF) differs from the most resistant cell line (PLC-PRF5) more than 28.5 fold. Furthermore, all cell lines, but Snu475, show a partial growth inhibition with positive GR_{max} values (see Figure 15A, Table 7). See also [12].

Figure 14C shows the effect of regorafenib on the hepatoma cell lines. At first glance, the cell lines seem to respond similarly as to sorafenib. However, some cell lines, such as Hep3B, HepG2, HLF, HuH7 and Snu475, show a statistically significant difference in response to regorafenib compared to sorafenib, with all of them being more responsive to regorafenib (see Figures 14A+C and 15, Table 7). Regorafenib displays a more heterogenous pattern of efficacy, inducing a slight cytotoxic effect at highest drug concentration in some cell lines ($GR_{max, R\text{-}Hep3B}$ -0.11, $GR_{max, R\text{-}HepG2}$ -0.29, $GR_{max, R\text{-}Snu398}$ -0.18), and a partial growth inhibition in others ($GR_{max, R\text{-}HLE}$ 0.12, $GR_{max, R\text{-}HLF}$ 0.22, $GR_{max, R\text{-}HuH1}$ 0.09, $GR_{max, R\text{-}HuH7}$ 0.10, $GR_{max, R\text{-}PLC\text{-}PRF5}$ 0.12) (see Figure 15A, Table 7). See also [12].

	Sorafenib			Lenvatinib			Regorafenib			Cabozantinib			RoS
	GR_{50}	GR_{max}	R	GR_{50}	GR_{max}	R	GR_{50}	GR_{max}	R	GR_{50}	GR_{max}	R	
Hep3B	1.7	-0.17	1	7.8	0.47	0	1.3	-0.11	1	3.1	0.34	1	1
HepG2	1.7	-0.25	1	6.7	0.16	0	1.2	-0.29	1	4.7	0.13	1	1
HLE	1.3	-0.12	1	0.9	0.19	1	1.2	0.12	1	5.2	0.13	0	0
HLF	2.3	-0.08	0	0.7	0.06	1	1.7	0.22	0	5.1	0.05	0	1
HuH1	2.3	-0.06	0	8.0	0.20	0	2.3	0.09	0	20	0.53	0	0
HuH7	2.4	-0.01	0	0.7	0.09	1	1.3	0.10	1	3.3	0.38	1	1
PLC-PRF5	2.9	0.11	0	20	0.64	0	2.6	0.12	0	10.3	0.38	0	0
Snu398	1.5	-0.16	1	1.0	0.47	1	1.2	-0.18	1	2.6	0.16	1	0
Snu475	2.8	-0.95	0	2.2	-0.95	1	1.5	-0.92	0	4.6	-0.98	1	1

*Table 7: **GR values for potency and efficacy.** This table shows the potency (GR_{50} in µM), efficacy (GRmax) and response (R) for each cell line and drug, as well as for one regorafenib over sorafenib (RoS) group. The response to the four TKIs is subgrouped in less sensitive (0) and more sensitive (1). The cut-off points for response chosen for each drug were determined by looking at the spread of GR_{50} values of the different cell lines, factoring in the steady state plasma concentration of each drug, as well as trying to achieve a 4:5 ratio for the two subgroups: 2µM for sorafenib, 1.5µM for regorafenib, 5µM for lenvatinib and cabozantinib. Cell lines with GR_{50} values below the cut-off point were declared more sensitive (R=1) and cell lines above this point less sensitive (R=0). For the RoS group, the cell lines were divided into being equally responsive to regorafenib and sorafenib (0) and showing a statistically significant difference in response to those two drugs, with all of them being more sensitive to regorafenib (1). Results are presented as the mean of at least three experiments. P<0.05 for all drugs by ANOVA. GRmax values can only be compared when the highest concentrations are equal. Therefore, they can be looked at when comparing how different cell lines react to one drug, but not when comparing how one cell line reacts to different drugs. Adapted from Sagmeister P, Daza J, et al. Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis. J Hepatocell Carcinoma. 2022;9:595-607 [12].*

Lastly, the effects of cabozantinib on the cell lines are shown in Figure 14D. Cabozantinib seems to be the overall least potent drug in comparison to the other TKIs' that have been examined in this study. Furthermore, it displays a very heterogenous pattern of response, with a wide spread of GR_{50} values. The most sensitive (Snu398, $GR_{50, C\text{-}Snu398}$ 2.6µM) and the least

sensitive (HuH1, $GR_{50,\ C\text{-}HuH1}$ >20 µM), differ by a factor greater than 7.6, respectively (see Figure 15, Table 7). Cabozantinib induces a partial growth inhibition in all cell lines but Snu475 (see Figures 14D and 15B, Table 7): $GR_{max,\ C\text{-}Hep3B}$ 0.34, $GR_{max,\ C\text{-}HepG2}$ 0.13, $GR_{max,\ C\text{-}HLE}$ 0.13, $GR_{max,\ C\text{-}HLF}$ 0.05, $GR_{max,\ C\text{-}HuH1}$ 0.53, $GR_{max,\ C\text{-}HuH7}$ 0.38, $GR_{max,\ C\text{-}PLC\text{-}PRF5}$ 0.38, $GR_{max,\ C\text{-}Snu398}$ 0.16 and $GR_{max,\ C\text{-}Snu475}$ -0.98. See also [12].

Figure 15B and Table 7 display the potency of each drug for the different cell lines. Sorafenib and regorafenib seem to be homogenously potent on all cell lines, while cabozantinib appears to be overall less potent in comparison. As mentioned above, lenvatinib displays a heterogenous pattern of responsiveness, with some drugs being very responsive and others less so. It should be noted that there are various ways to calculate the GR_{50} values. Firstly, we could graph a sigmoid curve of the mean measurements of all experiments and then determine the GR_{50} value graphically. Secondly, we could graph each experiment individually, determine a GR_{50} value graphically for each graph and then calculate the mean from all individual GR_{50} values. Or thirdly, we could calculate the GR_{50} value mathematically. In this work, we have used all three options and compared them to each other. For most cell line – drug combinations the GR_{50} values were quite similar. For those values that statistically differed from each other, we decided to use those values, that were graphically determined using the sigmoid curve of the mean values from all experiments. See also [12].

To further investigate if the differences in drug response are statistically significant, a one-way ANOVA was conducted for two concentrations per drug (sorafenib and regorafenib 2.5µM and 5µM, lenvatinib 6.67µM and 20µM, cabozantinib 10µM and 20µM). The one-way ANOVA showed statistically significant differences for all tested concentrations. However, this only proves that at least two cell lines show a statistically significant difference in response to one drug, but not which cell lines. Therefore, post-hoc tests were conducted. There appeared to be a statistically significant difference in response between Snu475 and every other cell line for all four drugs at their highest concentrations. In addition, the response of PLC-PRF5 vs. HLF at 20µM of cabozantinib, HepG2 vs. HuH7, PLC-PRF5, HLE, HLF and HuH1 as well as HLF vs. Hep3B and Snu398 at 5µM of regorafenib, and PLCPRF5 vs. HuH7, HepG2, Snu398, HLE and HLF at 20µM of lenvatinib, showed a statistically significant difference. See also [12].

5.3.2 According to cell lines

On the assumption that the cell lines we have utilized are potential surrogates for patients we determined how these responded to different drugs. Figures 16 A-I display the results of the cell viability assays according to each cell line. To compare the effect of the four drugs on one cell line, we looked at the GR_{50} values as markers for potency. However, the GR_{max} values for the individual drugs cannot be used to compare the drugs' effect on one cell line, given that GR_{max} values can only be compared to each other if the maximum concentrations are equal.

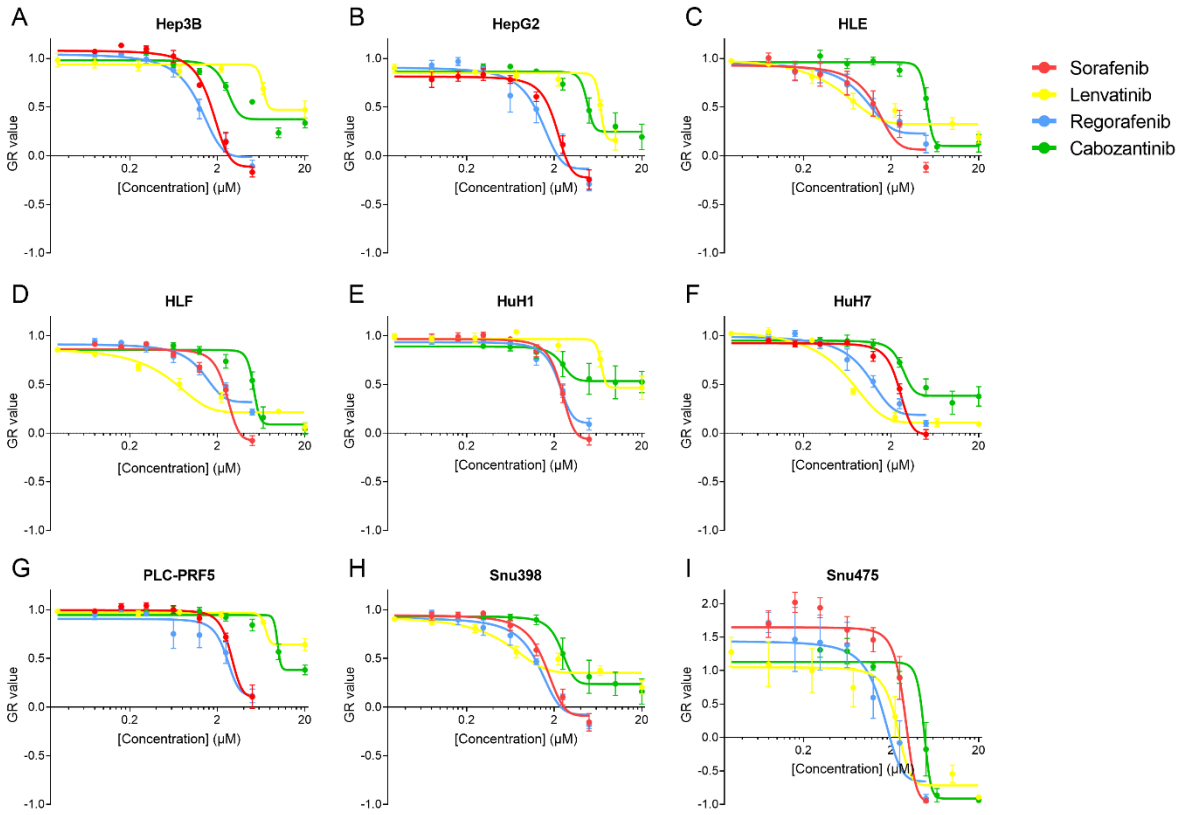

Figure 16: ***The effects of all the four tyrosine kinase inhibitors on one cell line****. Effects of sorafenib, lenvatinib, regorafenib and cabozantinib on* ***A*** *Hep3B,* ***B*** *HepG2,* ***C*** *HLE,* ***D*** *HLF,* ***E*** *HuH1,* ***F*** *HuH7,* ***G*** *PLC-PRF5,* ***H*** *Snu398 and* ***I*** *Snu475. The concentration ranges for each drug are as follows: sorafenib and regorafenib 0.08µM to 5µM in a 1:2 serial dilution, cabozantinib 0.31µM to 20µM in a 1:2 serial dilution, lenvatinib 0,02µM to 20µM in a 1:3 serial dilution. Results are presented as mean and SD values for at least three experiments. P<0.05 by ANOVA. Given that Snu475 displays GR values of up to 2.2 (including SD), the scale of the y-axis for this cell line reaches up to 2.2. Adapted from Sagmeister P, Daza J, et al. Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis. J Hepatocell Carcinoma. 2022;9:595-607 [12].*

Figures 16A + B show the effect of the four tyrosine kinase inhibitors on Hep3B and HepG2. These two cell lines react similarly to all four drugs. However, cabozantinib appears to be less potent on HepG2 than Hep3B ($GR_{50,C\text{-}HepG2}$ 4.7µM, $GR_{50,C\text{-}Hep3B}$ 3.1µM), while lenvatinib is more potent on HepG2 ($GR_{50,L\text{-}HepG2}$ 6.7µM, $GR_{50,L\text{-}Hep3B}$ 7.8µM). Both are least responsive to lenvatinib in comparison with the other seven cell lines. See also [12].

Figures 16C + D show the response of HLE and HLF to the different drugs. These two cell lines were derived from the same patient, with HLE being typically epithelial-like and HLF fibroblast-like but originating from hepatoma cells [70]. Therefore, the prediction could be

made that the two cell lines should react similarly to all drugs. However, there are some differences in response. HLE is an overall sensitive responder to all drugs, except cabozantinib ($GR_{50,L\text{-}HLE}$ 0.9µM, $GR_{50,R\text{-}HLE}$ 1.2µM, $GR_{50,S\text{-}HLE}$ 1.3µM). Similarly, HLF responded well to lenvatinib ($GR_{50,L\text{-}HLF}$ 0.7µM), regorafenib ($GR_{50,R\text{-}HLF}$ 1.7µM) and sorafenib ($GR_{50,S\text{-}HLF}$ 2.3µM), and cabozantinib was the least potent drug ($GR_{50,C\text{-}HLF}$ 5.1µM). When comparing HLE's and HLF's response to the four TKIs, all drugs seemed to be more potent on HLE than HLF. See also [12].

HuH1 and PLC-PRF5 are the overall least responsive cell lines (see Figures 16 E+G. Their reaction to sorafenib and regorafenib is almost identical, an effect observed for many cell lines, but Hep3B, HepG2, HLF, HuH7 and Snu475. HuH1 and PLC-PRF5 appear to be resistant to lenvatinib and cabozantinib. See also [12].

Figure 16F shows the effect of the four TKIs on HuH7. The most potent drug on HuH7 is lenvatinib with an GR_{50} value of 0.7µM, followed by regorafenib with 1.3µM, sorafenib with 2.4µM and cabozantinib with 3.3µM. Therefore, HuH7 is not most sensitive to sorafenib, which is often used as the TKI of first choice. Furthermore, the response of HuH7 to sorafenib and regorafenib showed a statistically significant difference, with HuH7 being more responsive to regorafenib. See also [12].

The effect of the TKIs on Snu398 is presented in Figure 16H. While lenvatinib seems to be highly potent on Snu398, it appears to be little efficient ($GR_{max,L\text{-}Snu398}$ 0.47). The reaction to sorafenib and regorafenib is similar, reaching a point of slight cytotoxicity at the highest concentration for both drugs (see Figure 15 and Table 7). See also [12].

Lastly, the reaction of Snu475 to the four TKIs is presented in Figure 16I. Snu475 is an unusual cell line. When looking at the IC_{50} values for Snu475, together with PLC-PRF5, is overall the least responsive cell line (see Table 8). Furthermore, the highest maximum inhibition at highest concentration for any of the drugs was only 65% at 5µM of sorafenib. For comparison, the next lowest maximum inhibition for sorafenib at 5µM was 85% for PLC-PRF5. Given that Snu475 is proliferating 12-times slower than the next slowest proliferating cell line (see Figure 13, Table 6), assuming an exponential growth, we decided on using the growth rate method to factor in the cell lines' proliferation rate in its response. Looking at the GR values, Snu475 is still not the most responsive cell line to any of the drugs, but it is the only one where all four TKIs induce complete cytotoxicity, with GR_{max} concentrations almost reaching -1 ($GR_{max,S\text{-}Snu475}$ = -0.95, $GR_{max,L\text{-}Snu475}$ = -0.95, $GR_{max,R\text{-}Snu475}$ = -0.92 and $GR_{max,C\text{-}Snu475}$ = -0.98, respectively; see Figure 17A). Furthermore, the GR_{50} concentrations of regorafenib ($GR_{50,R\text{-}Snu475}$ 1.5µM) and lenvatinib ($GR_{50,L\text{-}Snu475}$ 2.2µM) are lower than the GR_{50} concentration of sorafenib ($GR_{50,S\text{-}Snu475}$ 2.8µM). Once again, the potency for cabozantinib is the lowest with a GR_{50} concentration of 4.6µM. (see Figure 17B). See also [12].

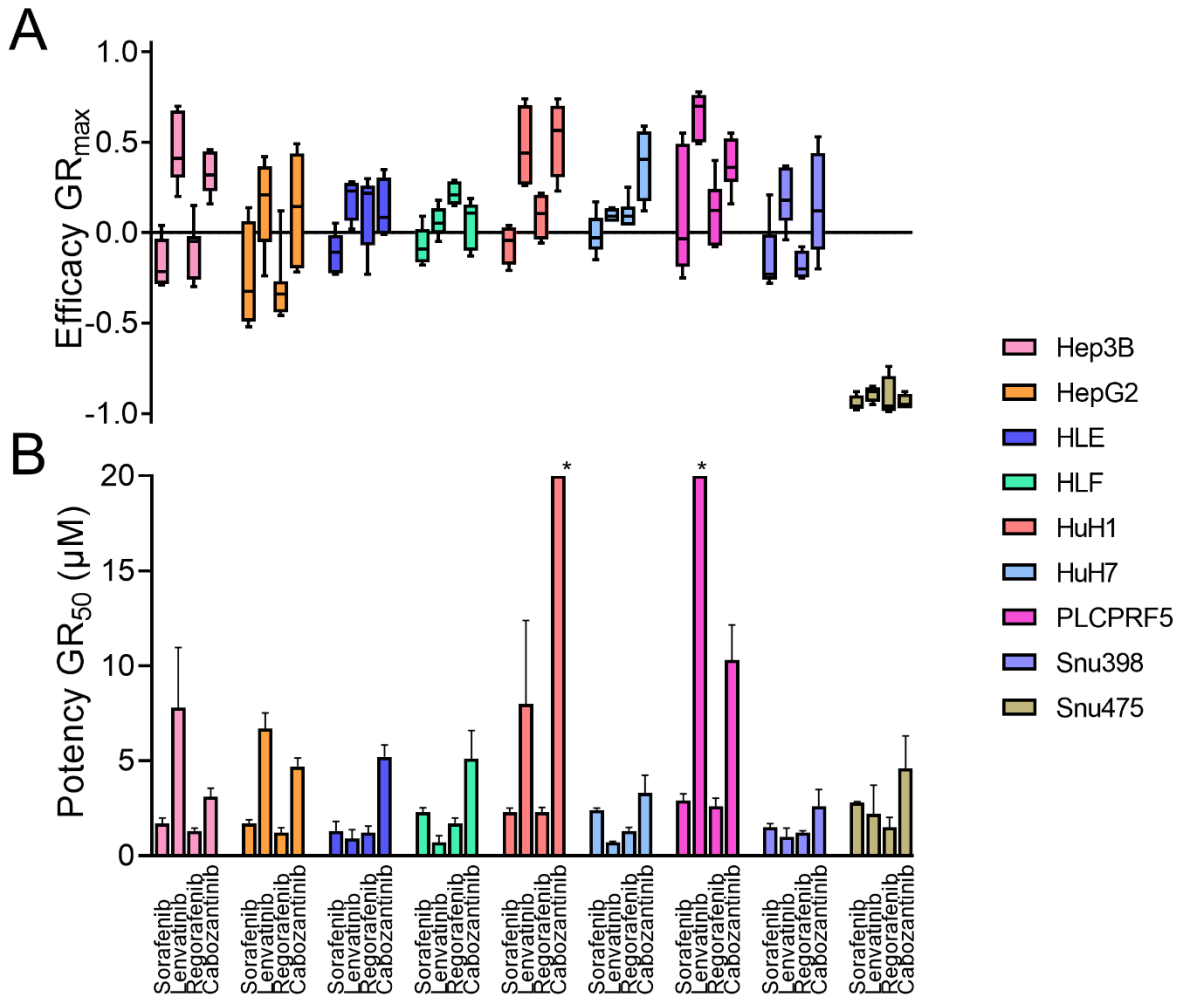

Figure 17: ***Potency and efficacy of the four TKIs on all nine hepatoma cell lines according to cell lines. A*** *Efficacy (GR_{max}) of sorafenib, lenvatinib, regorafenib and cabozantinib on all nine cell lines.* ***B*** *Potency (GR_{50} in µM) of sorafenib, lenvatinib, regorafenib and cabozantinib on all nine cell lines. Results are presented as the mean and SD values for at least three experiments. P<0.05 for all drugs by ANOVA. Adapted from Sagmeister P, Daza J, et al. Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis. J Hepatocell Carcinoma. 2022;9:595-607 [12].*

The efficacy of each drug on the nine cell lines is shown in Figure 17A. Some cell lines, such as HepG2 and PLC-PRF5 show a broad variability in efficacy, while others, such as HLE, HLF and HuH7, display a limited spread in GR_{max} values. As previously mentioned, Snu475 stands out by being the only drug inducing almost complete cytotoxicity, i.e. GR_{max} values near -1. See also [12].

Figure 17B displays the potency of each drug according to the cell lines. While some cell lines, such as HLE, HLF, HuH7 and Snu398, appear to be overall good responders to all drugs, others seem to be less responsive. Notably, both lenvatinib on PLC-PRF5 and cabozantinib on HuH1 did not induce a point, where 50% of the cells were no longer viable, thus the GR_{50} values were not reached at the maximum concentration. See also [12].

5.4 Cell cycle analysis

After discovering that the drugs cause a decrease in cell survival on the different cell lines, the question arose how these drugs intervene in the cell cycle to induce an inhibition on cell viability. Hence, a cell cycle analysis on two selected cell lines was conducted. By considering the proliferation rate, the cell lines' characteristics, as well as the variability of response to the different drugs, HuH7 and HLE were selected for cell cycle analysis. Both are proliferating rapidly and show an interesting spectrum of response in the cell viability assays. HuH7 is an AFP positive cell line, classified as S2 and grade G1. HLE is AFP negative, classified as S1 and undifferentiated (G4). Both cell lines are HBV negative and generated from a male patient [70, 72].

The cell cycle analysis involves separation of cells into four groups: sub-G1-, G1-, S- and G2/M- phases. G1 and G2 represent the "gaps" between DNA-replication and mitosis. In the G1-phase, cells are growing and preparing for the DNA-replication (S-phase). Here, the chromosomes are replicated from a haploid to a diploid genome. Afterwards, the cells continue growing (G2-phase), before entering the M-phase (mitosis), where cell division takes places. In the end, two identical daughter cells are produced. The sub-G1-events represent apoptotic cells, which exited the cell cycle at one point, i.e., they stopped proliferating. All phases are regulated by specific cyclin-dependent kinases and cyclin proteins. After cell staining with propidium iodide, the intensity of fluorescence is correlated with the amount of DNA contained in the cells, making it possible to distinguish the different phases of the cell cycle [93].

Sorafenib induces a time- and does-dependent G1-arrest in HuH7, as can be seen in Figure 18C. Correspondingly, a decrease in S- and G2/M-phases can be detected. When looking at the time kinetic of sub-G1 events, i.e., apoptosis, at 24h, 48h and 72 hours, at different concentrations of the drug, no increase in apoptosis is apparent (see Figure 18A). The percentage of cells going into apoptosis in the control group after 24 hours is 0.58%, being only 1.29% at the highest concentration after 72 hours. In comparison to HuH7's response to sorafenib, it induces a more discreet G1-arrest in HLE (see Figure 19C). HLE appears to have a higher natural apoptosis rate with approximately 3% of cells being apoptotic in the control group (see Figure 19A), vs. less than 1% for HuH7 in the control group. Similarly, only a slightly elevated number of cells went into apoptosis after 72 hours of incubation with 2.5µM sorafenib, when compared with the control group.

Regorafenib has a similar effect as sorafenib on both cell lines. Figures 18+19 show that regorafenib induces a G1-arrest, that is more discreet in HuH7 than in HLE, and in general less distinct when compared to sorafenib. Unlike sorafenib, an increase of sub-G1-events can be detected in HuH7, with approximately 1% of cells being apoptotic in the control group and

almost 6% after treatment with 2.5µM of regorafenib after 72 hours. No such increase in sub-G1 events was apparent when looking at HLE.

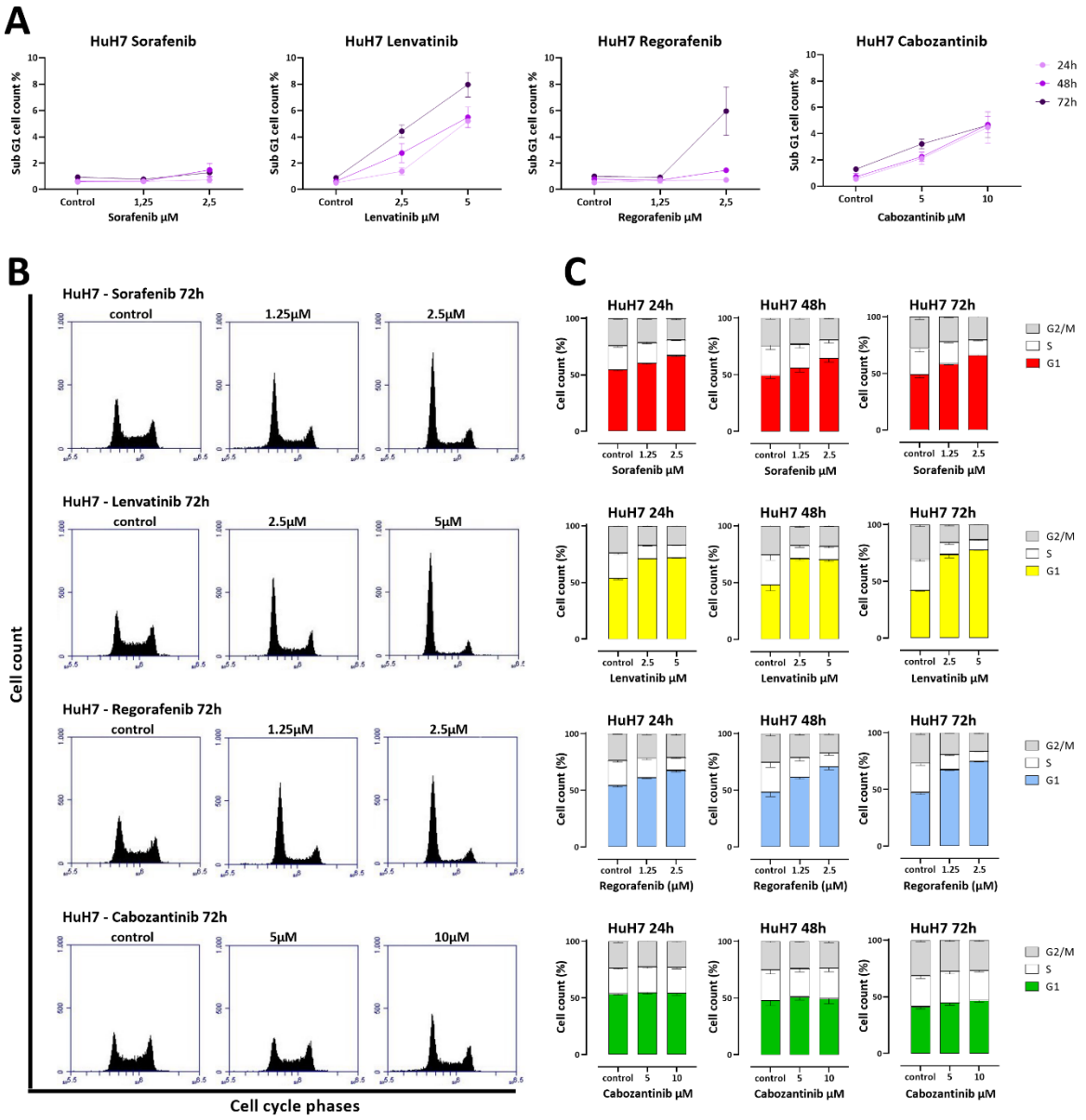

*Figure 18: **Cell cycle analysis of all four tyrosine kinase inhibitors on HuH7.** HuH7 was incubated with sorafenib (1.25µM and 2.5µM), lenvatinib (2.5µM and 5µM), regorafenib (1.25µM and 2.5µM) or cabozantinib (5µM and 10µM) for 24, 48 or 72 hours. **A** Sub-G1 events upon incubation with each drug at concentration 0 and two different concentrations, as stated above, are shown as the apoptosis rate at each concentration. **B** Differences in cell distribution between different drugs and concentrations. **C** Cell cycle analysis was performed using fluorescence activated cell sorting, and the results were compared to a control. (p<0.05, ANOVA) Results are presented as the mean and SD values for at least three experiments.*

Figures 18+19 show that lenvatinib induced a G1-arrest in both cell lines, with the accompanying decrease in S- and G2/M-phases. Additionally, lenvatinib caused a continuously increasing number of cells going into apoptosis (see Figure 18A + 19A). After treatment with 5µM of lenvatinib for 72 hours 8% of HuH7 and 10% of HLE were apoptotic.

To summarize, sorafenib, regorafenib and lenvatinib all induced a G1-arrest, which is most distinct in cells treated with regorafenib and lenvatinib. In HLE, there was a corresponding decrease in cells in the S-phase, while the G2/M-phase almost remained constant. While Sorafenib did not cause a significant number of cells to go into apoptosis, regorafenib showed a slight, and lenvatinib a clear increase in sub-G1-events in a dose- and time-dependent manner.

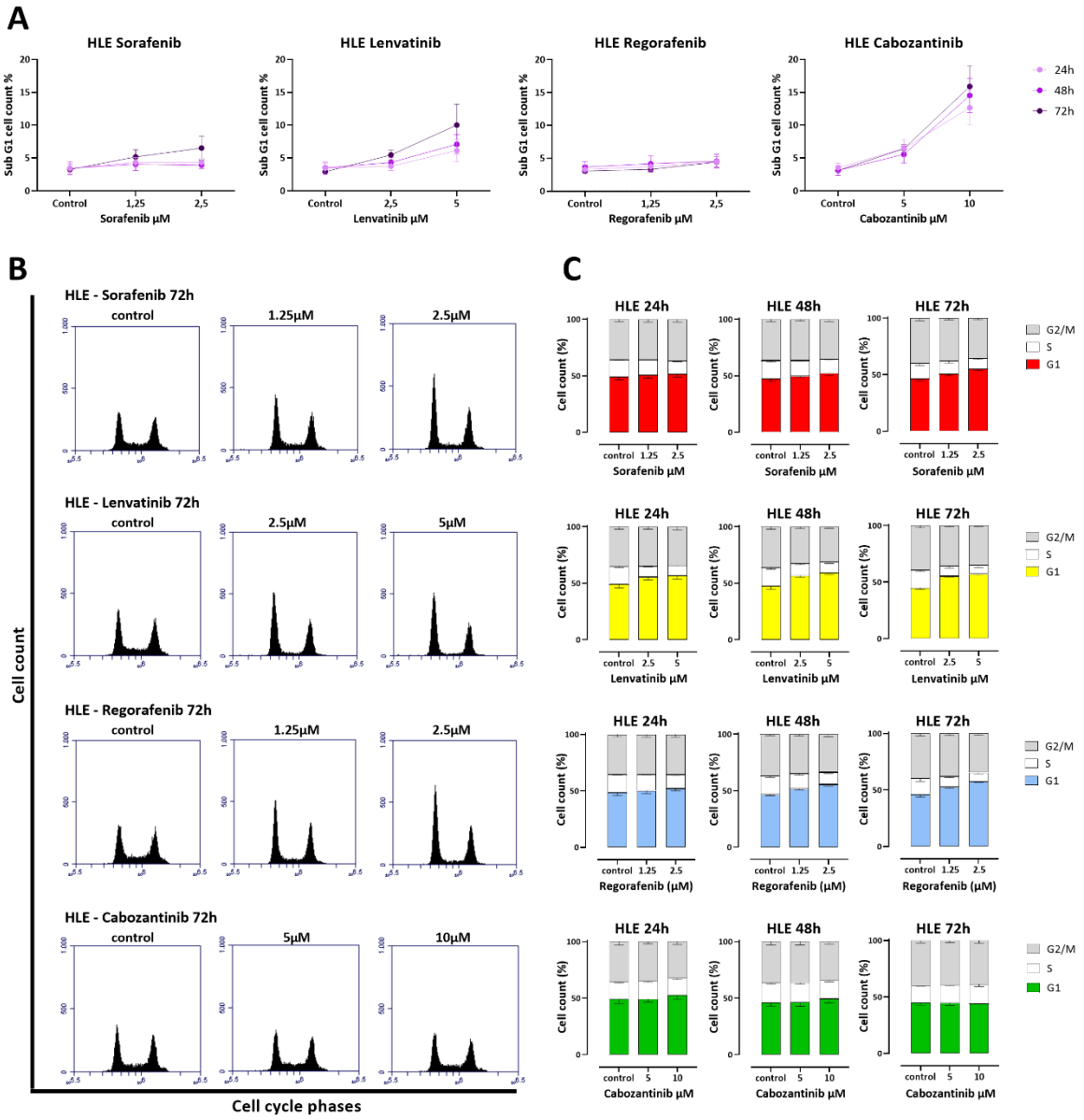

Figure 19: ***Cell cycle analysis of all four tyrosine kinase inhibitors on HLE.*** *HLE was incubated with sorafenib (1.25µM and 2.5µM), lenvatinib (2.5µM and 5µM), regorafenib (1.25µM and 2.5µM) or cabozantinib (5µM and 10µM) for 24, 48 or 72 hours.* ***A*** *Sub-G1 events upon incubation with each drug at concentration 0 and two different concentrations, as stated above, are shown as the apoptosis rate at each concentration.* ***B*** *Differences in cell distribution between different drugs and concentrations.* ***C*** *Cell cycle analysis was performed using fluorescence activated cell sorting, and the results were compared to a control. (p<0.05, ANOVA) Results are presented as the mean and SD values for at least three experiments.*

Figures 18+19 show how cabozantinib interferes in the cell cycle of HuH7 and HLE. Cabozantinib had no significant effect on any of the phases of the cell cycle in either of the two cell

lines. There was a slight increase in cells going into the G1-phase with increasing doses and duration of drug treatment for both cell lines, but this increase was so little, that it cannot be declared significant. Two experiments, out of six repetitions, showed a tendency towards a G1-arrest at stimulation with 5µM of cabozantinib at all time points, followed by a pronounced G2-arrest at the highest concentration of 10µM of cabozantinib (see Figure 20). These two experiments were excluded from further analysis (see discussion). All repetitions revealed an increasing percentage of sub-G1-events in a time- and dose-dependent manner (see Figures 18A + 19A). The percentage of cells going into apoptosis was more pronounced in HLE (16% sub-G1-events at 10µM cabozantinib after 72 hours), compared to HuH7 (4.6% sub-G1-events at 10µM cabozantinib after 72 hours).

6. DISCUSSION

6.1 *In vitro* study

Given the availability of various hepatoma cell lines in our laboratory, our experience in working with cell lines and this work's restricted time, we decided to conduct an *in vitro* study without a validation *in vivo*. While working with cell lines brings numerous advantages, such as the possibility of infinitive reproducibility, easy availability, cost-efficiency, the option for genetic modifications, better control of conditionality and minimizing confounding factors, there are also some major limitations, such as two-dimensional growth and failure to show typical characteristics of the parental tumour [94].

Two aspects that are crucial for understanding the *in vivo* effectiveness of a drug are pharmacodynamics and pharmacokinetics. Pharmacodynamics answers the question what effect a drug has on an organism. Pharmacokinetics looks at what a metabolism does with the drug, that is its absorption into the blood circulation, its distribution in the body, its metabolism, and its elimination. Possible ways of elimination include metabolic, renal and bile [95-97].

With our *in vitro* study, we can make some assumptions of how a drug works. We can see that all four tyrosine kinase inhibitors lead to a dose and time dependent decrease in cell viability and that they effect the cells through a G1-arrest or an increased number of cells going into apoptosis. However, we cannot say what effect the drug will have on the human body. How much of the drug is reaching the liver tissue after liberation, absorption, biotransformation, distribution, and elimination processes? What adverse effects will occur at the concentration used in these experiments? Will these adverse effects be so insufferable, they will limit the drug usage? These are all questions that must be answered to understand the usability of a drug in a clinical setting but cannot be answered by conducting *in vitro* experiments.

Luckily, there have already been various studies investigating human pharmacodynamics and pharmacokinetics of the four tyrosine kinase inhibitors used in this study [90, 98-100], which can be looked at for comparison and completion of our findings.

6.2 Hepatoma cell lines do not represent heterogeneity of HCC

HCC is an incredibly heterogenous and complex type of disease. Therefore, it is quite difficult to find a small group of hepatocellular carcinomas that will adequately represent HCC in its entirety and makes precision medicine even more difficult [9, 68].

There are approximately 30 known hepatoma cell lines available. This small number is not sufficient for representing the complex heterogeneity of HCC, especially given that they were

all generated in the 20th century and the aetiologies of HCC are constantly changing over time [101, 102].

As stated above, approximately 50% of HCCs can be attributed to liver cirrhosis due to a previously acquired chronic Hepatitis B infection and another 25% are caused by liver cirrhosis due to a chronic HCV infection. The remaining 25% are caused by liver cirrhosis of other aetiologies, chronic HBV and HCV infections without accompanying liver cirrhosis, excessive alcohol intake, aflatoxin B1-exposure and NAFLD, just to name a few [2, 15]. Approximately 73% of all patients diagnosed with an HCC are male and 27% female [1]. For comparison, 55.6% of the cell lines used in this study are HBs-Ag positive, none were tested positive for HCV, and all were generated from male patients [69-74]. Therefore, the hepatoma cell lines used in these experiments, do not represent the heterogeneity of HCCs' aetiology today, without even taking the genetic nature of an HCC into account. Female patients, patients whose tumour's aetiology is based on a previously acquired HCV infection and other aetiologies, such as alcohol intake or the rising risk factor of metabolic syndrome and diabetes leading to NAFLD, are not represented at all by these nine cell lines or most available hepatoma cell lines.

Upon deciding to conduct an *in vitro* study, what can be done to improve the heterogeneity of the HCC samples which are to be investigated? Various research groups have been working on generating hepatocellular carcinoma models generated from patients, such as the Liver Cancer Model Repository (LIMORE) by Qiu et al. which generated 50 HCC models from 49 patients [9], or patient derived organoids. Organoids are a three-dimensional replicate of the original tumour that keeps the original tumour's histological, genetic, and transcriptomic features [103, 104]. Organoids are established by digesting the primary tumour, plating the tumour cells in basement membrane extract and then cultivating them in a special growth medium, plied with various inhibitory and growth factors [102]. Given that organoids keep most features of its parental tumour, they are an ideal tool for an individualized drug testing to predict the patient's specific response to a drug [94].

While these methods show huge potential for precision medicine and better representation of today's aetiologies of HCC, the rate of success is a problem: Qiu et al. reported a success rate of 55% for generating their cancer cell models as well as a successful establishment of a cell model from a Grad II tumour, which previously failed to be established as organoids [9]. Nuciforo et al. and Broutier et al. reported a success rate of 20-30% for generating organoids [104, 105]. Furthermore, organoids lack an accurate immune system and blood vessels, and they take a long time to mature [106].

While there are a lot of exciting new approaches to *in vitro* studies, the work with cell lines remains the backbone of *in vitro* experiments, due to their reliability and broad availability.

6.3 Drug Assay

6.3.1 Methods

Prior to the conduction of the drug assays, we have decided upon using a longer duration of drug incubation (144h) in comparison to most other drug assays (24-72h) [9, 10]. This decision was made due to the week to month long intact of the tyrosine kinase inhibitors in the clinical setting.

Additionally, we also decided on using the GR_{50} and GR_{max} values as metrics for potency and efficacy, instead of the most used and well established IC_{50} and E_{max} values. The GR method adjusts the cell survival after drug treatment for a cell line's velocity of proliferation [76]. In the clinical setting, a patient is typically treated with a TKI for weeks to months. Hence, the drug has sufficient time to take effect on the tumour cell, even if the tumour is proliferating slowly. In an *in vitro* setting with a maximum incubation period of 72 hours, the drug has only little time to take effect on all cell lines, the ones that are proliferating quickly, as well as the ones proliferating slowly. By adjusting the cell survival for the proliferation time, we can see the actual effect the drug has on one cell line, independently of its proliferation time. While it was supposedly the right decision for us to adjust our data with the GR method, given the huge span in the various cell lines' proliferation indexes, it makes it considerably more difficult to compare our data to other research groups'. However, it is not entirely correct to compare two datasets which have both used the half maximal inhibitory concentration (IC_{50}), unless they have both used the same assay, same time of drug incubation and equal drug concentrations. And even then, the conditions in which the assays were conducted and the person conducting the assays would still differ from each other, making it difficult to compare them properly [107].

Furthermore, when we defined the parameters for conducting our drug assay, we were more interested in how the cells react to smaller concentrations of the drug, rather than choosing a bigger maximum concentration. Therefore, we chose a concentration range of 0.08µM to 5µM for sorafenib and regorafenib. In retrospect, the concentration ranges for both drugs could have started with a maximum concentration of 10µM, given that both drugs' steady state concentrations are greater than 5µM and some cell lines did not reach complete cell death at the highest concentration that we have decided on using.

We have also chosen individual concentration ranges for each drug, instead of using one range for all four drugs. The decision to use the same concentration ranges for each drug, would have made the comparison of one cell line's reaction to the four drugs considerably easier. However, the decision was made to use the optimal, individual range for each drug, given that the dosage of the different TKIs in the clinical setting is also not equal, and we wanted to see how the drug works in its optimal concentration range.

6.3.2 Results

There are numerous *in vitro* studies on the effect of tyrosine kinase inhibitors on hepatoma cell lines by various research groups. I would like to focus on two, a project by Qiu et al. [9] and one by Caruso et al. [10], because even though both had different aims and endpoints to their studies, the drug assays they conducted are similar to the one we carried out. The results of our drug assay, compared with the one conducted by Qiu et al. and Caruso et al. can be seen in Table 8. See also [12].

	Sorafenib						**Lenvatinib**					
Cell line	**GR_{50}**	**IC_{50}**	**IC_{50}-Q**	**GI_{50}-C**	**R**	**SS**	**GR_{50}**	**IC_{50}**	**IC_{50}-Q**	**GI_{50}-C**	**R**	**SS**
Hep3B	1.7	1.4	3.3	6.3	1	11.6	7.8	6.7	20	10	0	0.8
HepG2	1.7	1.4	5.3	2.5	1	11.6	6.7	6.6	20	10	0	0.8
HLE	1.3	0.5	3.8	3.7	1	11.6	0.9	0.4	20	10	1	0.8
HLF	2.3	1.6	8.4	6.9	0	11.6	0.7	0.4	20	10	1	0.8
HuH1	2.3	1.9	7.7	7.0	0	11.6	8.0	7.2	20	10	0	0.8
HuH7	2.4	1.5	5.2	5.7	0	11.6	0.7	0.6	3.2	3.0	1	0.8
PLC-PRF5	2.9	2.7	7.2	10	0	11.6	>20	>20	20	10	0	0.8
Snu398	1.5	1.1	9.2	5.1	1	11.6	1.0	0.5	20	10	1	0.8
Snu475	2.8	3.8	20	8.1	0	11.6	2.2	7.4	20	10	1	0.8
	Regorafenib						**Cabozantinib**					
Cell line	**GR_{50}**	**IC_{50}**	**IC_{50}-Q**	**GI_{50}-C**	**R**	**SS**	**GR_{50}**	**IC_{50}**	**IC_{50}-Q**	**GI_{50}-C**	**R**	**SS**
Hep3B	1.3	1.0	1.5	10	1	8.1	3.1	2.4	7.4	9.8	1	3.3
HepG2	1.2	1.1	3.6	10	1	8.1	4.7	3.1	11.5	10	1	3.3
HLE	1.2	0.6	4.4	10	1	8.1	5.2	3.0	20	10	0	3.3
HLF	1.7	1.2	9.5	10	0	8.1	5.1	4.8	12.9	10	0	3.3
HuH1	2.3	1.6	20	10	0	8.1	>20	3.2	10.8	10	0	3.3
HuH7	1.3	0.8	3.5	6	1	8.1	3.3	2.4	5.7	7.5	1	3.3
PLC-PRF5	2.6	1.7	8.0	10	0	8.1	10.3	5.8	13.0	10	0	3.3
Snu398	1.2	0.9	10.3	10	1	8.1	2.6	2.4	20	7.8	1	3.3
Snu475	1.5	3.3	20	10	0	8.1	4.6	5.7	13.7	10	1	3.3

*Table 8: **Comparison of our research results with similar experiments.** This table shows the results from our experiments (GR_{50} and IC_{50} values), as well as the results from Qiu et al. (IC_{50}-Q) [9] and Caruso et al. (GI_{50}-C) [10], both of which conducted experiments similar to ours, for each cell line and drug. All GR/IC/GI values are shown in µM. The response (R) is subgrouped into less sensitive (0) and more sensitive (1), as previously described. Furthermore, the steady state concentrations (SS in µM) of each drug after multiple doses over a specific duration of time is shown. SS_S = 11.55µM after multiple doses of 400mg bid sorafenib p.o. over a duration of 28 days [86], SS_L = 0.78µM after multiple doses of 12mg lenvatinib p.o. for four weeks [87], SS_R = 8.08µM after multiple doses of 160mg regorafenib p.o. daily [85], and SS_C = 3.27 after multiple doses of 140mg cabozantinib for 29 days [90]. Adapted from Sagmeister P, Daza J, et al. Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis. J Hepatocell Carcinoma. 2022;9:595-607 [12].*

While we have decided on using the GR method by Hafner et al. with its accompanying GR_{50} and GR_{max} values as metrics for potency and efficacy [76], Qiu et al. used the popular IC_{50} value and Caruso et al. one GI_{50} value, which is the IC_{50} value that has been corrected for the cell count at the start of drug treatment [108]. Moreover, we used an essay with a longer duration of drug treatment (144h), while Caruso et al. and Qiu et al. both used shorter essays (48-72h). Given that the assays that have been used, the duration of drug treatment and the metrics for potency are all different, a direct comparison between these three studies is not possible. What we can do is look at the trends and overall results of all three studies. All three, show a dose-dependent decrease in cell survival for most cell lines and drugs. In comparison to our results, some of Qiu et al.'s and Caruso et al.'s results for regorafenib, lenvatinib and

cabozantinib did not meet the IC/GI_{50} values (see Table 8), possibly either due to lower maximum drug concentrations or shorter assay durations. While we can see that all cell lines show an apparent dose dependent decrease in cell survival to all drugs, Qiu et al. and Caruso et al. both describe the effect of the four tyrosine kinase inhibitors, sorafenib, lenvatinib, regorafenib and cabozantinib, among the littlest compared to all other substances that have been tested in their studies. This is a very interesting finding, given that most other substances that have been tested in their studies are not approved for clinical use of hepatocellular carcinoma even though some have been tested for that exact purpose [109, 110]. If our results and various others [82, 83, 91, 111] had not shown a good efficacy of those four substances on hepatoma cell lines *in vitro*, we would have to ask ourselves why the TKIs' effect *in vitro* is less than *in vivo*. However, we know that the four TKIs cause a decrease in cell survival on HCC cell lines *in vitro* and *in vivo*, and it is therefore most likely that the combination of a longer drug incubation and higher drug concentrations explains why we can observe a stronger effect of decrease in cell survival in our results, when compared to Qiu et al.'s and Caruso et al.'s [9, 10]. See also [12].

		Sorafenib	**Lenvatinib**	**Regorafenib**	**Cabozantinib**	**Overall**
GR_{50} vs. IC_{50}	Correlation	linear	linear	neither	neither	linear
	Pearson's r	0.858*	0.955*	0.345	0.186	0.744*
	Spearman's Rho r	0.928	0.819	0.749*	0.678*	0.877
	P-value	0.003	<0.001	0.020	0.045	<0.001
GR_{50} vs. $IC_{50}Q$	Correlation	cubic	neither	neither	neither	neither
	Pearson's r	-0.005	0.274	0.453	-0.103	0.302
	Spearman's Rho r	-0.118	0.481	0.385	0.050	0.321
	P-value	0.763	0.190	0.307	0.898	0.059
GR_{50} vs. $GI_{50}C$	Correlation	linear	neither	neither	neither	neither
	Pearson's r	0.836*	0.274	0.208	0.384	0.299
	Spearman's Rho r	0.815	0.481	0.070	0.782*	0.243
	P-value	0.005	0.190	0.858	0.013	0.153
GR_{50} vs. GR_{max}	Correlation	cubic	cubic	neither	neither	cubic
	Pearson's r	-0.178	0.462	0.294	0.355	0.430
	Spearman's Rho r	0.370	0.639	0.368	0.227	0.428*
	P-value	0.327	0.064	0.330	0.557	0.009

*Table 9: **Correlation coefficients (r) between GR_{50} and IC_{50}, $IC_{50}Q$, $GI_{50}C$ or GR_{max} values for all four TKIs as well as overall.** GR_{50}, IC_{50} and GR_{max} values are metrics from this work's results, $IC_{50}Q$ from Qiu et al.'s and $GI_{50}C$ from Caruso et al.'s works. This table shows the type of correlation (linear, cubic, or neither) according to curve fitting, Pearson's and Spearman's Rho correlation coefficient and the p-value, for each drug and overall, for all drugs. If there is a linear correlation between two factors, we are looking at Pearson's correlation coefficient; if there is a cubic correlation between two factors, or no clear cubic or linear correlation, we are looking at the more robust Spearman's Rho correlation coefficient. Both correlation coefficients (Pearson and Spearman's Rho) can be interpreted according to Cohen: 0.1>r shows no, 0.1 ≤ r < 0.3 a small, 0.3 ≤ r <0.5 a medium and r ≥ 0.5 a strong correlation between the two factors [79]. * Marks a statistically significant correlation between the two factors, i.e., p<0.05. Adapted from Sagmeister P, Daza J, et al. Comparative response of HCC cells to TKIs; modified in vitro testing and descriptive expression analysis. J Hepatocell Carcinoma. 2022;9:595-607 [12].*

When comparing the three datasets, we can see various correlations with different strengths, between our results and the other two datasets, as can be seen in Table 9. There is an especially strong correlation between our results and Caruso et al.'s for sorafenib (Pearson's correlation coefficient (r) 0.836, p-value = 0.005). Additionally, we can see small (cabozantinib, Spearman's Rho-r = 0.227, p-value = 0.557) to strong correlations (lenvatinib, Spearman's Rho-r = 0.639, p-value 0,064) between the potency (GR_{50}) and efficacy (GR_{max}) of one drug. Due to the high p-values these correlations cannot be declared statistically significant and are potentially chance findings. See also [12].

Furthermore, some cell lines, e.g. HLE, showed a homogenous responsiveness to the various drugs. This could lead us to the assumption that some cell lines – and in foresight patients – are high responders to most TKIs, and therefore a standard therapy without individualization would be justifiable. However, most other cell lines displayed a diversified response to the four TKIs that have been tested in this study. While sorafenib and regorafenib seemed to induce a homogenous response in all cell lines with the span of GR_{50} values being close together, lenvatinib and cabozantinib showed a more heterogenous pattern with some cell lines responding well to one drug and others less so. This is a further indication that the search for genetic or transcriptomic biomarkers, especially for those drugs that induce a heterogenous pattern of response, is necessary to predict and improve a patient's outcome in a clinical setting. See also [12].

Another notably interesting observation to be made was the response of the cell lines to regorafenib when comparing to sorafenib. Most cell lines' reaction was almost identical to sorafenib and regorafenib. This is not particularly surprising, given that the structural formular of regorafenib differs from sorafenib's by only one additional fluor atom [52], sorafenib and regorafenib target almost identical kinds of receptor tyrosine kinases and other target points [36], and regorafenib is only approved for clinical use in hepatocellular carcinoma if the patient has previously been treated with sorafenib [112]. However, some cell lines showed a statistically significant difference in response to the two drugs, with all of them being more sensitive to regorafenib. For these cell lines it would be particularly interesting if there were any markers to predict their response to one of the two. And we must ask, if regorafenib should find its place as a first- or second-line therapy, independently of a previous sorafenib use. See also [12].

6.4 Cell cycle analysis

6.4.1 Methods

Before conducting the cell cycle analysis, the decision had to be made which and how many cell lines to perform the analysis on, which and how many different concentrations for each drug to use, and at which and at how many time points to stop and evaluate the analysis.

In the end, the two cell lines HuH7 and HLE were used for the cell cycle analysis. Both responded overall well to all four TKIs in the drug assays and are the fastest proliferating cell lines we have used in these experiments, as can be seen in Figure 13 and Table 6. However, we did non evaluate any cell lines that proliferated relatively quickly but did not respond well to all drugs, such as HuH1 or PLC-PRF5. A cell cycle analysis on one of these cell lines could have possibly shown us a difference in effect on the cell cycle for one of the drugs that could have explained why the drug's effect on these cell lines was less when compared to others. Furthermore, we also did not investigate the effect of the four drugs on Snu475, a unique cell line that responded with a cytotoxic effect to all drugs that have been examined in this study. While it would have been interesting to see in what phase of the cell cycle the different drugs induce this cytotoxicity in Snu475, this line was proliferating so slowly that we believed the cell cycle analysis not to be an option, given that the time points for evaluation were chosen after a shorter period of drug incubation when compared to the drug assay.

Secondly, we decided on two concentrations for each drug: 1.25µM and 2.5µM for sorafenib and regorafenib, 2.5µM and 5µM for lenvatinib, and 5µM and 10µM for cabozantinib. While the first drug concentration was supposed to be high enough to show the cells' reaction to the drug but low enough that not all cells were completely affected, the second concentration should induce the drug's maximum effect on the cell line's cell cycle. In hindsight, the maximum concentration could have been set closer to the drug assay's maximum concentrations for each drug. However, we aspired to not have the two concentrations too far apart to observe a trend rather than two absolutes. Therefore, the two concentrations per drug were decided upon as mentioned above.

24, 48 and 72 hours after drug incubation, the analysis was stopped, and the results evaluated. These three timepoints showed a trend of effect with the passage of time. Prior to conducting the cell cycle analysis, we elected to choose a seven-day incubation with the drug. However, we feared that with a longer drug incubation the number of cells going into apoptosis would have been falsely high and therefore obscuring the validity of our results.

The strategy for conducting a cell cycle analysis is almost infinitive. We have decided on designs that seemed reasonable to use for our research aim. Even though the time frame and drug concentration of the drug assay and cell cycle analysis were not equal we can still make

some assumptions on what effect one drug has on different cell lines and how this drug achieves this effect by interfering in one cell line's cell cycle by choosing two representative examples for the entirety of all cell lines.

6.4.2 Results

Sorafenib and regorafenib showed a similar mode of interference in the cell cycle in both cell lines by causing a G1-arrest, with an accompanying decrease in the S- and G2/M-phases. While regorafenib also caused an increased number of cells going into apoptosis, no such increase in sub-G1-events could be detected when looking at sorafenib. When comparing the cell lines' reaction to the two drugs, we can see that the G1-Arrest is more pronounced in HuH7 for both drugs. The G1-arrest in HuH7 caused by regorafenib is more distinct than the one caused by sorafenib. This could support our findings in the drug assay that showed a statistically significant difference in response of HuH7 to sorafenib and regorafenib with HuH7 being more responsive to regorafenib. In HLE we can observe a slight G1-arrest for both sorafenib and regorafenib, with no apparent difference in strength. Similarly, the response of HLE to sorafenib and regorafenib in the drug assay was almost identical.

Lenvatinib induced a distinct dose-dependent G1-arrest as well as an increasing number of cells going into apoptosis in both cell lines. In our drug assay, we could observe that four cell lines (HuH7, HLE, HLF and Snu398) responded well with GR_{50} values ≤ 1.0µM and four cell lines (Hep3B, HepG2, HuH1 and PLC-PRF5) responded very little to lenvatinib. Unfortunately, both HuH7 and HLE belong to the first group of cell lines that responded well to lenvatinib. In hindsight, it would have been interesting to perform the cell cycle analysis on one of the cell lines that responded minimally to lenvatinib, compare the results to HuH7 or HLE and evaluate if there is any difference in interference in the cell cycle of the two groups of cell lines.

Cabozantinib lead to a slight increase of cells going into the G1-phase, which could possibly be a coincidental observation, as it was not redeemed statistically significant. Additionally, it caused a pronounced time- and dose-dependent increase in number of cells going into apoptosis. This find corresponds with our results from the drug assay which showed cabozantinib to be the least potent drug out of the four examined TKIs. However, we know that cabozantinib causes a significant prolongment of life for patients with advanced HCC by 2.2 months (10.2 months cabozantinib vs 8.0 months placebo), as has been shown by Abou-Alfa in 2018 [8]. This leads us to the question if cabozantinib is less effective *in vitro* when compared with *in vivo*. However, other research groups, such as Xiang et al. reported that cabozantinib displayed a good effectiveness on various hepatoma cell lines and induced a dose-dependent G1-arrest, accompanied by an increased number of cells going into apoptosis [91]. It is possible that the concentrations of cabozantinib that we have chosen for conducting our drug

assay and cell cycle analysis were simply not high enough and therefore cabozantinib appeared to be having little effect on hepatoma cell lines.

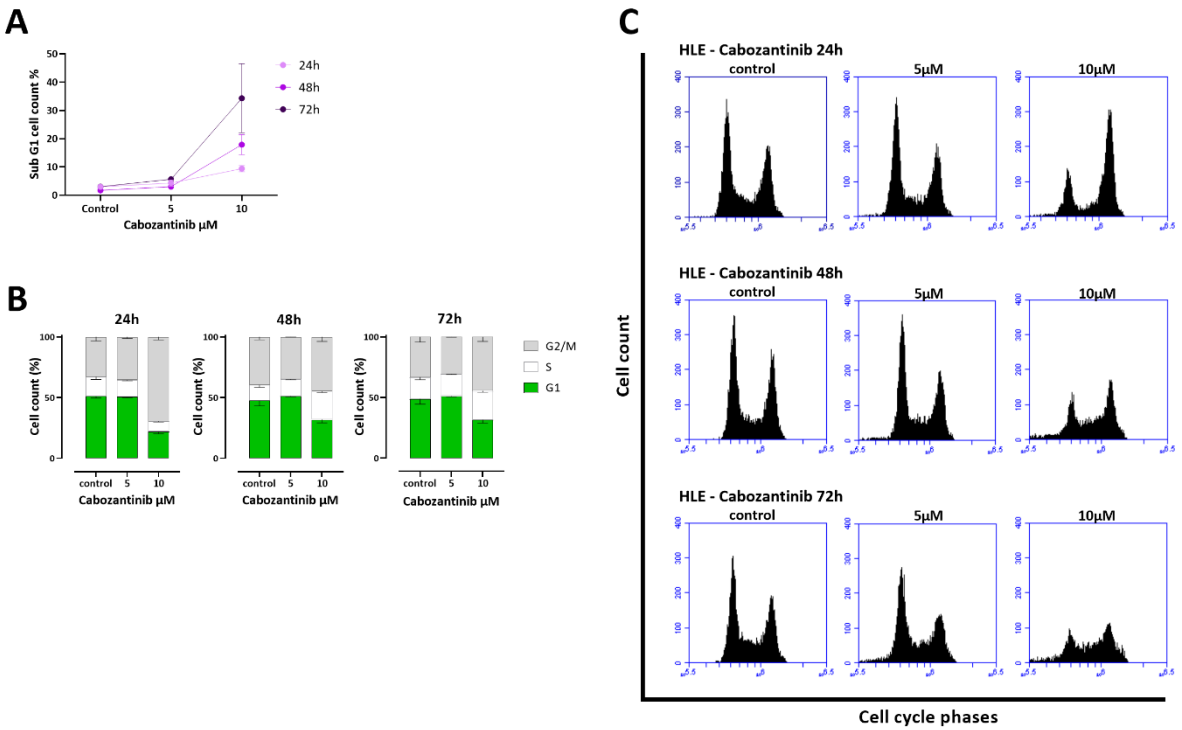

Figure 20: ***Cell cycle analysis of cabozantinib on HLE showing a G2-arrest.*** *HLE was incubated with cabozantinib (5µM and 10µM) for 24, 48 or 72 hours.* ***A*** *Sub-G1 events upon incubation with cabozantinib at concentration 0, 5µM and 10µM. are shown as the apoptosis rate at each concentration.* ***B*** *Cell cycle analysis was performed using fluorescence activated cell sorting, and the results were compared to a control. P<0.05, ANOVA.* ***C*** *Differences in cell distribution between concentrations and time points. Results are presented as the mean and SD values for two experiments.*

As stated before, two individual experiments from the cell cycle analysis with cabozantinib were taken out of the final analysis. These two experiments showed a pronounced G2-arrest in combination with sub-G1 events up to 40% at the highest concentration. While we need to consider that these results might be valid, there are reasons why we decided on not including them in the final results: Firstly, the experiments that showed distinct G2-arrest (see Figure 20) were not reproducible. Secondly, a distinct G2-arrest would not have correlated with our findings in the drug assay that have been repeated multiple times. Thirdly, the results seemed to be inconsistent in a time- and dose-dependent manner: the G2-arrest of the HLE cells was most distinct after 24 hours. After 48 and 72 hours there was still a G2-arrest detectable but less pronounced. Additionally, there seemed to be no difference between the untreated cells and those that were treated with 5µM cabozantinib. Therefore, we assume that these two measurements were incorrect.

However, we need to ask ourselves, why we have gotten these results and how we can explain this error. One possibility, for the faulty measurement could have been that cabozantinib does not tolerate thawing and re-freezing. We have usually prepared a 10µM drug-medium

solution for all drugs prior to the cell cycle analysis. This solution was used for up to three individual experiments after which any remnants were discarded. In-between experiments the solution was thawed and afterwards put back into a -80°C storage. Conspicuously, none of the two experiments with the distinct G2-arrest occurred after the solution was prepared immediately before executing the experiments.

6.5 Endpoint of this study

It was this study's aim to directly compare the effect of the four tyrosine kinase inhibitors, sorafenib, lenvatinib, regorafenib and cabozantinib on different hepatoma cell lines, and further to detect if there was an imminent need for the search for biomarkers. Furthermore, we evaluated how the four drugs interfere in a cell line's cell cycle and if we can detect any differences in mode of action. All four TKIs are already approved for clinical use on advanced hepatocellular carcinomas and have therefore been tested and evaluated in the past. However, most research has not focused on these four drugs but rather used one of the four to compare to a new, potential drug option [84, 113] or has conducted a study with numerous agents rather than focus on the optimal concentration ranges for the four TKIs [9, 10].

We have found a differential response of the different cell lines to one drug, as well as a differential response of one cell line to the four TKIs. All four TKIs lead to a dose- and time-dependent decrease in cell survival. These results neither come as a surprise, nor are they ground-breaking. However, we did find that most cell lines respond homogenously well to regorafenib and sorafenib, some cell lines respond better to lenvatinib than to sorafenib, some cell lines respond better to regorafenib than to sorafenib, and most respond minimally to cabozantinib. The cell cycle analysis showed that most of the four TKIs induce an interference in a cell line's cell cycle by either causing a G1-arrest or increasing the number of cells going into apoptosis.

7. SUMMARY

The treatment options for hepatocellular carcinomas at an advanced stage, that is BCLC C cancer stage [29], are still limited and unsatisfactory, the sole intent of treatment being a prolongment of life. The gold standard for the treatment of HCC at an advanced stage are currently the use of immune-oncology therapies, such as atezolizumab plus bevacizumab [4] or durvalumab plus tremelimumab [114]. If the patient shows any contraindications for the use of immuno-oncology-based treatment options or the tumour shows a progression of disease under said therapies, the well-established TKIs are the next choice of therapy in the treatment algorithm for advanced HCC [32]. We believe that the four TKIs, sorafenib, lenvatinib, regorafenib and cabozantinib, alone or in combination with CPIs will remain the backbone of treatment for advanced HCCs in the future. Unfortunately, there are no established biomarkers to guide us to the TKI with the best possible outcome for an individual patient and the TKIs' line of treatment was determined by empirical evidence only [3]. Therefore, we have decided on conducting an *in vitro* viability assay to determine the differential responsiveness to the different TKIs followed by a cell cycle analysis to observe the various means of interference of a drug in a cell line's cell cycle.

We have conducted a drug assay assessing the reaction of nine hepatoma cell lines with different clinical, histological, and transcriptomic features (Hep3B, HepG2, HLE, HLF, HuH1, HuH7, PLC-PRF5, Snu398 and Snu475) to the four TKIs in seven different concentrations, ranging from 0.03 to 20µM. A DMSO-medium solution was used as a control group. Cell survival was estimated after six days of drug incubation. To adjust our values for the varying proliferation rates of the various cell lines, we have decided on using the growth rate method by Hafner et al. with its accompanying GR_{50} and GR_{max} values as values for potency and efficacy [76]. Afterwards, we have conducted a cell cycle analysis on two cell lines to investigate how the four TKIs interfere with the cell lines' cell cycle and evaluated the effect of the drugs on the cell cycle after 24, 48 and 72 hours of drug incubation.

The nine cell lines showed a pronounced variability of response after treatment with one of the four TKIs. While sorafenib and regorafenib induced a homogenously potent effect on all cell lines and a slight cytotoxic effect at the maximum concentration for most cell lines, the cell lines' reaction to cabozantinib was overall less responsive. Lenvatinib appeared to be the most interesting drug, separating the cell lines into two groups, depending on their potency: the first group, consisting of HLE, HLF, HuH7 and Snu398, displaying GR_{50} values ≤ 1µM; and the second group, consisting of Hep3B, HepG2, HuH1 and PLC-PRF5, displaying GR_{50} values ≥ 6.7 µM. By comparing the reaction of one cell line to all drugs, we have found some cell lines, such as Hep3B, HepG2, HLF, HuH7 and Snu475, to show a significant difference in response

to sorafenib and regorafenib, with all of them being more sensitive to regorafenib. When comparing our results with those of other research groups, e.g. Qiu et al.'s [9] or Caruso et al.'s [10], it appears that we have found the drugs to be most potent on HCC cell lines, which could be due to our longer drug incubation time.

The search for possible biomarkers by combining our results with the cell lines' transcriptomic data would have been an interesting continuation of our research. We continued this project after the completion of this work in cooperation with another research group from Mannheim University, Germany. We have found some markers that appear to distinguish the cell lines, and in foresight patients, intro three treatment groups: those that respond well to sorafenib, regorafenib and cabozantinib; those that respond well to lenvatinib; and those that respond better to regorafenib than sorafenib. Given that these results are merely based on the response of nine hepatoma cell lines to the four TKIs with no *in vivo* validation, they need to be treated carefully and further validated in subsequent studies. The results of this project were published in Journal of Hepatocellular Carcinoma in July 2022 [12].

Compendiously, we have found a diversified responsiveness of the nine different hepatoma cell lines to the four TKIs. Additionally, we have identified cell lines that respond better to the second-line treatment option regorafenib than the first-line treatment option sorafenib, and some cell lines which are more responsive to lenvatinib than its fellow first-line therapy option sorafenib. These findings prompt us to further evaluate potential biomarkers for the four TKIs to individualize and optimize the therapy for hepatic malignancy. These results should lead us to critically question and reconsider the treatment paradigm for advanced HCC.

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Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Comparative response of nine hepatoma cell lines to the four tyrosine kinase inhibitors, sorafenib, lenvatinib, regorafenib and cabozantinib.

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