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Establishing a Human Liver-on-a-Chip Model in Vitro

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Abstract

Drug-induced liver injury (DILI) remains a major challenge in drug development, highlighting the need for human-relevant preclinical models. This study aimed to establish and functionally validate a perfused human Liver-on-a-Chip model using differentiated HepaRG cells and immortalized human liver sinusoidal endothelial cells (LSECs) cultured on the Dynamic42 dual-channel microfluidic platform.

Differentiated HepaRG cells and LSECs were seeded on opposite sides of a porous membrane to generate compartmentalized co-cultures under continuous vascular perfusion. Model functionality was evaluated by exposure to staurosporine as a positive control and the clinically relevant antibiotics levofloxacin and meropenem. Cellular responses were assessed by daily brightfield microscopy and quantification of lactate dehydrogenase (LDH) release.

Stable co-cultures were successfully maintained throughout the experimental period. Staurosporine induced pronounced morphological deterioration and markedly increased LDH release in both cellular compartments, demonstrating the model's ability to detect acute cytotoxicity. In contrast, levofloxacin and meropenem caused neither significant morphological alterations nor increased LDH release compared with untreated controls, consistent with their low intrinsic hepatotoxic potential at clinically relevant concentrations.

In conclusion, a robust and reproducible HepaRG–LSEC Liver-on-a-Chip model was successfully established. The platform provides a physiologically relevant in vitro system for investigating acute drug-induced cytotoxicity and represents a promising tool for improving preclinical drug safety assessment while supporting the reduction of animal experimentation.

Zusammenfassung

Arzneimittelinduzierte Leberschäden (Drug-Induced Liver Injury, DILI) stellen eine große Herausforderung in der Arzneimittelentwicklung dar und verdeutlichen den Bedarf an humanrelevanten präklinischen Modellen. Ziel dieser Arbeit war die Etablierung und funktionelle Validierung eines perfundierten humanen Liver-on-a-Chip-Modells unter Verwendung differenzierter HepaRG-Zellen und immortalisierter humaner lebersinusoidaler Endothelzellen (LSECs) auf der Dynamic42-Dual-Channel-Mikrofluidikplattform. Differenzierte HepaRG-Zellen und LSECs wurden auf gegenüberliegenden Seiten einer porösen Membran ausgesät, um kompartimentalisierte Kokulturen unter kontinuierlicher vaskulärer Perfusion zu etablieren. Die Funktionalität des Modells wurde durch Behandlung mit Staurosporin als Positivkontrolle sowie den klinisch relevanten Antibiotika Levofloxacin und Meropenem untersucht. Zelluläre Reaktionen wurden mittels täglicher Hellfeldmikroskopie sowie durch Quantifizierung der Freisetzung von Laktatdehydrogenase (LDH) bewertet.

Während des gesamten Versuchszeitraums konnten stabile Kokulturen erfolgreich aufrechterhalten werden. Staurosporin führte zu ausgeprägten morphologischen Veränderungen sowie einer deutlich erhöhten LDH-Freisetzung in beiden Zellkompartimenten und bestätigte damit die Fähigkeit des Modells, akute Zytotoxizität nachzuweisen. Im Gegensatz dazu verursachten Levofloxacin und Meropenem im Vergleich zu unbehandelten Kontrollen weder relevante morphologische Veränderungen noch eine erhöhte LDH-Freisetzung, was mit ihrem geringen intrinsischen hepatotoxischen Potenzial bei klinisch relevanten Konzentrationen übereinstimmt.

Zusammenfassend konnte ein robustes und reproduzierbares HepaRG-LSEC-Liver-on-a-Chip-Modell erfolgreich etabliert werden. Die Plattform stellt ein physiologisch relevantes In-vitro-Modell zur Untersuchung akuter arzneimittelinduzierter Zytotoxizität dar und bietet eine vielversprechende Grundlage für eine verbesserte präklinische Sicherheitsbewertung von Arzneimitteln bei gleichzeitiger Reduktion von Tierversuchen.

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1 Introduction

1.1 The Liver: Central Role in Human Physiology

The liver is a central metabolic organ that plays a crucial role in maintaining systemic homeostasis through nutrient metabolism, detoxification, protein synthesis, and immune regulation (Alamri, 2018). Its strategic anatomical position and dual blood supply from the hepatic artery and portal vein enable continuous exposure to dietary nutrients, microbial products, and xenobiotics absorbed from the gastrointestinal tract (Abdel-Misih & Bloomston, 2010; Alamri, 2018). Approximately 70–75% of hepatic blood flow originates from the portal vein, supporting efficient first-pass metabolism and clearance of absorbed compounds (Alamri, 2018).

Hepatocytes coordinate carbohydrate, lipid, and protein metabolism to regulate systemic energy balance. They store glucose as glycogen and release it during fasting, synthesize and oxidize fatty acids, and produce the majority of plasma proteins, including albumin and coagulation factors (Alves-Bezerra & Cohen, 2017; Han et al., 2016; Korolova et al., 2023). The liver also performs the urea cycle to detoxify ammonia generated during amino acid catabolism, thereby preventing systemic toxicity (Alamri, 2018). These tightly regulated metabolic processes highlight the liver’s essential role in maintaining physiological stability.

Beyond metabolic regulation, the liver represents the primary organ for xenobiotic biotransformation. Hepatocytes metabolize endogenous waste products and pharmaceuticals through coordinated phase I and phase II pathways, largely mediated by cytochrome P450 (CYP) enzymes (Alamri, 2018). These reactions convert lipophilic compounds into more water-soluble metabolites suitable for elimination via bile or urine. The liver’s high metabolic activity and continuous exposure to xenobiotics, however, also render it particularly susceptible to toxic injury (Gu & Manautou, 2012).

In addition to parenchymal hepatocytes, the liver contains specialized non-parenchymal cell populations that contribute to immune surveillance and tissue homeostasis. Liver sinusoidal endothelial cells (LSECs) form a highly permeable interface that facilitates molecular exchange and regulates immune tolerance, while Kupffer cells patrol the sinusoidal lumen and coordinate inflammatory responses (Puri & Sonawane, 2025; Trefts et al., 2017). Together, these metabolic and immunological functions position the liver as an integrated metabolic–immune interface essential for whole-body homeostasis.

Given its central role in metabolism, detoxification, and immune regulation, disruption of hepatic function can rapidly lead to systemic imbalance, inflammation, or organ failure (Alamri, 2018). This functional complexity underscores both the vulnerability

of the liver to drug-induced injury and the challenge of accurately reproducing hepatic physiology in experimental models (Wang et al., 2023).

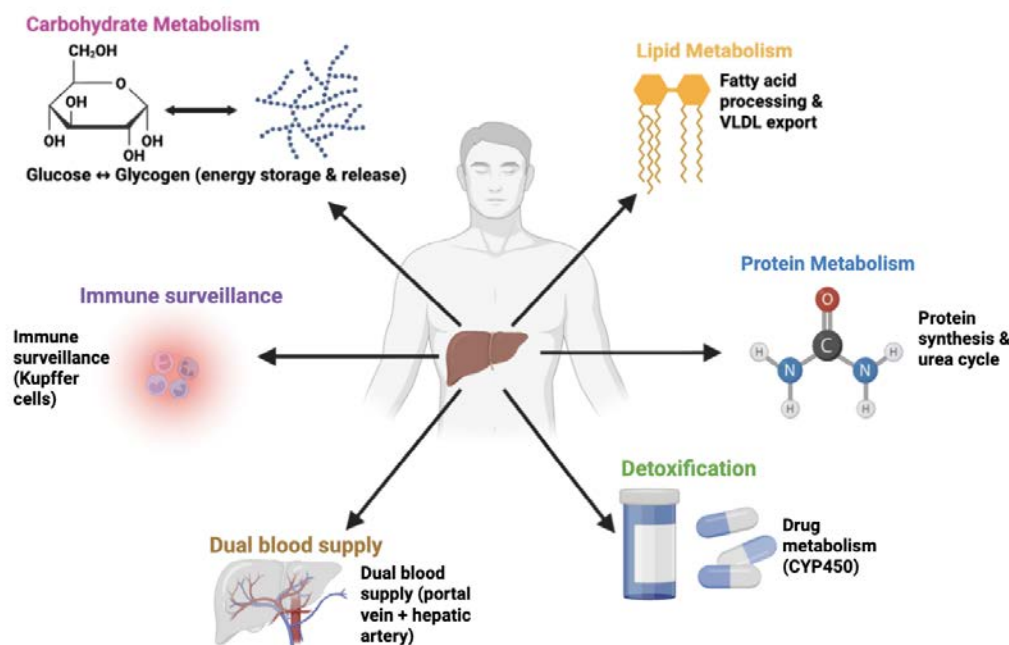


Figure 1: Overview of the major physiological functions of the human liver. The illustration summarizes the liver's central roles in carbohydrate, lipid, and protein metabolism, xenobiotic detoxification, immune surveillance, and its dual blood supply. These integrated functions reflect the organ's high metabolic activity and continuous exposure to circulating compounds, which contribute to its susceptibility to toxic injury. Illustration created with BioRender.com.

1.2 Anatomy and Cellular Architecture of the Liver

The functional unit of the liver is the hepatic lobule, a highly organized microanatomical structure centered around a central vein and bordered by portal triads composed of the portal vein, hepatic artery, and bile duct (Huppert & Iwafuchi-Doi, 2019). As illustrated in Fig. 2(a), blood flows from the periportal region toward the central vein through specialized capillaries known as sinusoids, where venous and arterial blood mix and enable continuous exposure of hepatocytes to nutrients, oxygen gradients, and xenobiotics (Wang et al., 2023; Wierling, 2014). This spatial organization establishes a dynamic microenvironment essential for metabolic processing and detoxification.

Within the lobule, hepatocytes are arranged in polarized plates extending between

portal triads and the central vein, forming the structural framework of the parenchyma (Huppert & Iwafuchi-Doi, 2019). Their basolateral membrane interfaces with sinusoidal blood, while the apical membrane forms bile canaliculi that ensure directional bile secretion (Hanley et al., 2017). The sinusoidal flow pattern depicted in Fig. 2(b) establishes oxygen and nutrient gradients along the porto-central axis, resulting in functional zonation that influences susceptibility to toxic injury (Berasain & Avila, 2014; Wang et al., 2023). This zonated architecture is a key determinant of region-specific drug metabolism and damage.

At higher resolution, the sinusoidal microenvironment (Fig. 2(c)) reveals the intimate spatial relationship between hepatocytes, liver sinusoidal endothelial cells (LSECs), and the space of Disse. The sinusoidal lumen is lined by LSECs, a specialized endothelial subtype characterized by fenestrations arranged in sieve plates and the absence of a continuous basement membrane (Sørensen et al., 2015). Together with hepatocytes and the intervening space of Disse, LSECs form a highly permeable exchange interface that facilitates rapid molecular transfer between blood and parenchyma (Sørensen et al., 2015). In addition to mediating nutrient exchange, LSECs regulate immune cell trafficking and contribute to tolerogenic antigen presentation, thereby maintaining immunological balance (Shetty et al., 2018).

Kupffer cells reside within the sinusoidal lumen and provide continuous immune surveillance by clearing pathogens, debris, and damaged cells (English et al., 2021; W. Li et al., 2022). Through cytokine release and metabolic crosstalk with hepatocytes and LSECs, they influence inflammatory signaling and oxidative stress responses (W. Li et al., 2022). In the space of Disse, hepatic stellate cells (HSCs) regulate extracellular matrix composition and maintain structural integrity of the sinusoidal interface (Kamm & McCommis, 2022). Upon injury, HSC activation drives fibrotic remodeling, further altering hepatic microarchitecture (Kamm & McCommis, 2022). Cholangiocytes lining the bile ducts coordinate bile modification and transport, completing the structural and functional integration of hepatobiliary flow (Ghonem et al., 2015).

Collectively, the coordinated organization of hepatocytes, LSECs, Kupffer cells, stellate cells, and cholangiocytes—summarized schematically in Fig. 2—forms a tightly regulated multicellular system in which vascular flow, extracellular matrix structure, and cellular polarity determine metabolic efficiency and immune balance (Huppert & Iwafuchi-Doi, 2019). Reproducing this spatial complexity and multicellular communication remains a central challenge for experimental liver models.

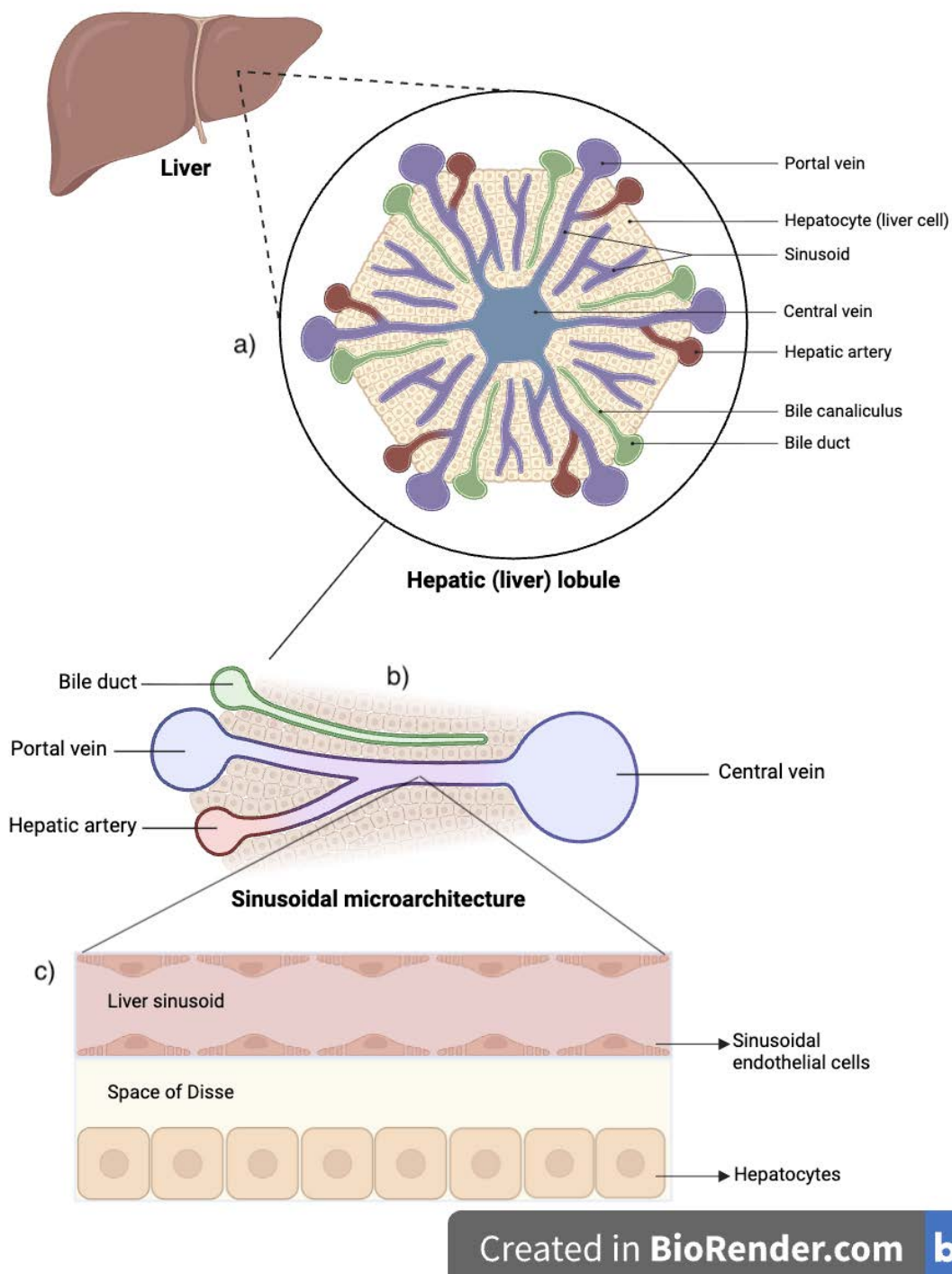


Figure 2: Schematic overview of the hepatic lobule and sinusoidal microarchitecture. (a) Anatomical organization of the hepatic lobule, including hepatocytes, sinusoids, portal triads, central vein, hepatic artery, portal vein, and bile ducts. (b) Sinusoidal vascular architecture illustrating portal inflow from the hepatic artery and portal vein and venous outflow toward the central vein. (c) Magnified view of the sinusoidal microenvironment, highlighting liver sinusoidal endothelial cells (LSECs), the space of Disse, and underlying hepatocytes, which together establish the structural and functional interface for molecular exchange, metabolic regulation, and immune interactions. Illustration created with BioRender.com.

1.3 Drug-Induced Liver Injury (DILI)

Drug-induced liver injury (DILI) represents a major challenge in clinical medicine and pharmaceutical development, as it remains one of the leading causes of acute liver failure and post-marketing drug withdrawal (Kim & Naisbitt, 2016). DILI refers to liver damage caused by exposure to drugs, herbal products, or other xenobiotics and encompasses a broad clinical spectrum ranging from asymptomatic elevations of liver enzymes to fulminant hepatic failure (Alempijevic et al., 2017; Andrade et al., 2019). DILI is generally classified into intrinsic and idiosyncratic forms. Intrinsic DILI, exemplified by acetaminophen toxicity, is predictable, dose-dependent, and reproducible in experimental systems (Andrade et al., 2019). In contrast, idiosyncratic DILI (iDILI) occurs unpredictably in a small subset of individuals and is largely dose-independent. Its development is influenced by genetic susceptibility, immune factors, and environmental conditions (Segovia-Zafra et al., 2021). Although less frequent, iDILI accounts for a substantial proportion of severe clinical cases and remains particularly difficult to predict due to the lack of reliable biomarkers (Chen et al., 2014).

Mechanistically, DILI arises from complex interactions between metabolic activation, oxidative stress, mitochondrial dysfunction, cholestasis, and immune-mediated processes (Saleh et al., 2025; Yan et al., 2018). Cytochrome P450 (CYP)-dependent biotransformation can generate reactive intermediates that promote excessive production of reactive oxygen species (ROS). The resulting oxidative stress induces lipid peroxidation, protein oxidation, and DNA damage, thereby compromising mitochondrial integrity and hepatocyte viability (Villanueva-Paz et al., 2021; Zhang et al., 2018).

Mitochondrial dysfunction represents a critical amplification step in hepatocellular injury. Loss of mitochondrial membrane potential, opening of the mitochondrial permeability transition pore, and depletion of intracellular ATP disrupt cellular energy homeostasis and can ultimately trigger apoptosis or necrosis, depending on the severity of the damage (Ramachandran et al., 2018; Villanueva-Paz et al., 2021). In parallel, oxidative stress disrupts intracellular calcium homeostasis and activates pro-apoptotic signaling pathways such as c-Jun N-terminal kinase (JNK), further exacerbating hepatocellular injury (Ramachandran & Jaeschke, 2019; Wu et al., 2017).

Reactive metabolites may also form covalent drug-protein adducts that function as neoantigens, thereby initiating adaptive immune responses through T-cell activation and cytokine release (Teschke & Danan, 2023; Villanueva-Paz et al., 2021). This immune response contributes to hepatocyte injury in genetically susceptible individuals and may help explain the idiosyncratic nature of many DILI cases, particularly those associated with specific human leukocyte antigen (HLA) haplotypes (Daly, 2025; Roth et al., 2022).

In addition, impairment of bile acid transporters such as the bile salt export pump (BSEP) and multidrug resistance-associated proteins (MRPs) can disrupt bile acid homeostasis (Garzel et al., 2019). Consequently, impaired bile acid transport may lead to the intracellular accumulation of toxic bile acids in hepatocytes, which promotes inflammatory signaling and further contributes to liver injury (M. Li et al., 2017; Qu et al., 2018; Vitale et al., 2023).

Beyond hepatocytes, non-parenchymal cells also play a crucial role in shaping DILI progression. Kupffer cells regulate inflammatory responses and contribute to both tissue injury and repair processes (Tasnim et al., 2021), while liver sinusoidal endothelial cells (LSECs) maintain hepatic microcirculation and respond to toxic stress by modulating cytokine release and adhesion molecule expression (Fernández-Iglesias & Gracia-Sancho, 2017). The interplay between parenchymal and non-parenchymal cells therefore critically influences the severity and outcome of liver injury.

Overall, DILI represents a multifactorial and patient-specific process that integrates metabolic stress, mitochondrial dysfunction, immune activation, and disrupted bile acid homeostasis. The complexity of these mechanisms highlights the need for advanced human-relevant in vitro systems capable of recapitulating multicellular interactions, metabolic competence, and immune-associated signaling in order to improve the prediction and mechanistic understanding of drug-induced hepatotoxicity.

1.4 Current Challenges in Liver Research Models

Despite decades of use, animal models often fail to reliably predict human drug-induced liver injury (DILI), largely due to fundamental interspecies differences in drug metabolism, immune regulation, and cytochrome P450 enzyme activity (McGill & Jaeschke, 2019; Van Norman, 2019). In particular, idiosyncratic DILI cannot be reliably reproduced in rodents, as these reactions depend on complex metabolic and immune interactions that are species-specific and poorly recapitulated in animal systems (McGill & Jaeschke, 2019). Combined with ethical considerations and translational limitations, these discrepancies substantially reduce the predictive value of animal studies for the evaluation of human liver safety (Olson et al., 2000; Panwar et al., 2021).

Conventional two-dimensional (2D) hepatocyte cultures represent another widely used approach but suffer from rapid functional deterioration. Within days, hepatocytes lose metabolic activity, polarity, and liver-specific gene expression due to downregulation of transcriptional networks essential for maintaining hepatic identity (Kiamehr et al., 2019; Panwar et al., 2021). Furthermore, static 2D systems lack physiological extracellular matrix architecture, biomechanical cues, and fluid flow, all of which are critical for sustaining stable hepatocyte function. Consequently, key structural features such as

bile canaliculi formation and proper transporter localization are not maintained (Godoy et al., 2013; Panwar et al., 2021).

Three-dimensional spheroid and organoid models better mimic the native liver microenvironment by promoting more physiologically relevant cell–cell and cell–matrix interactions and improving the expression of liver-specific markers (Bell et al., 2016; Xu, 2021). However, these systems remain limited by heterogeneity in size and morphology, diffusion constraints that generate hypoxic or necrotic cores, and restricted long-term reproducibility (Bell et al., 2016; Xu, 2021). Stem-cell-derived organoids frequently exhibit fetal-like metabolic profiles, limiting their ability to model adult hepatic drug metabolism (Ma et al., 2023). Additionally, reliance on biological matrices such as Matrigel introduces batch variability and hinders standardization for translational or high-throughput applications (Fatehullah et al., 2016; Panwar et al., 2021).

A critical limitation shared by many conventional *in vitro* systems is the insufficient integration of non-parenchymal cell types. *In vivo*, hepatocytes function within a multicellular sinusoidal niche comprising liver sinusoidal endothelial cells (LSECs), Kupffer cells, and hepatic stellate cells (Ma et al., 2023; Trefts et al., 2017). The absence of LSECs disrupts endothelial–parenchymal communication and nutrient exchange, while omission of Kupffer and stellate cells prevents adequate modeling of inflammation, immune activation, and fibrotic remodeling—processes central to many forms of DILI (Bale et al., 2014; Panwar et al., 2021). Without this coordinated multicellular environment, current systems cannot faithfully reproduce the complexity of human liver physiology and drug responses (Ma et al., 2023).

As a result, numerous compounds that appear safe in preclinical testing later cause clinically relevant hepatotoxicity (Walker et al., 2020). Traditional *in vivo* and *in vitro* models fail to simultaneously capture metabolic competence, immune interactions, and microenvironmental regulation that drive intrinsic and idiosyncratic liver injury (McGill & Jaeschke, 2019). Even commonly used biomarkers such as ALT and AST lack sufficient sensitivity and mechanistic specificity for early toxicity detection (Dirven et al., 2021). The persistent disconnect between preclinical evaluation and clinical outcome underscores the urgent need for more predictive human-relevant systems.

Collectively, these limitations highlight the necessity of advanced *in vitro* platforms that combine multicellular architecture, physiological perfusion, controlled shear stress, and long-term functional stability. Microfluidic technologies capable of recreating dynamic hepatic microenvironments therefore represent a promising step toward bridging the translational gap between experimental models and human liver physiology (Ma et al., 2023; Panwar et al., 2021; Xu, 2021).

1.5 Liver-on-a-Chip Technology

Liver-on-a-Chip (LoC) platforms are microphysiological systems developed to recapitulate essential structural and functional aspects of the human liver by combining engineered microenvironments with living hepatic cells. Their primary objective is to reproduce key sinusoidal characteristics, including controlled perfusion, oxygen and nutrient gradients, and spatial organization of parenchymal and non-parenchymal cells (Beckwitt et al., 2018). Using microfabrication and microfluidic technologies, LoC devices generate defined microchannels that enable continuous media flow, thereby providing dynamic shear stress and regulated mass transport conditions that more closely resemble *in vivo* hepatic physiology than static culture systems (Qiu et al., 2023). By integrating multicellular layers or compartmentalized architectures separated by porous membranes (Figure 3), these systems aim to restore physiological cell–cell communication and functional polarization within a controlled three-dimensional microenvironment (Dalsbecker et al., 2022). As a result, LoC platforms have been shown to maintain metabolic activity, bile canaliculi formation, and zonation-like behavior that are typically lost in conventional culture formats (Deguchi & Takayama, 2022). Compared with traditional 2D and 3D models, Liver-on-a-Chip systems provide improved physiological relevance through dynamic perfusion, which stabilizes liver-specific functions by preventing nutrient depletion, limiting metabolite accumulation, and preserving oxygen gradients characteristic of hepatic microcirculation (Ferrari & Rasponi, 2021). Sustained flow conditions support long-term cytochrome P450 (CYP) expression and continuous albumin and urea production, thereby maintaining metabolic competence over extended culture periods (Ferrari & Rasponi, 2021; Qiu et al., 2023). Importantly, the incorporation of multiple hepatic cell types enhances predictive capacity by enabling direct and paracrine interactions between hepatocytes, endothelial cells, stellate cells, and macrophages—interactions that are essential for modeling inflammatory responses, extracellular matrix remodeling, and drug-induced stress (Dalsbecker et al., 2022). Recent studies, including the Dynamic42 platform, demonstrated that perfused LoC systems can discriminate between hepatotoxic and non-hepatotoxic compounds with higher sensitivity than static models (Kaden, Graf, et al., 2023).

A critical design consideration for LoC models is the choice of hepatic and endothelial cell types. HepaRG cells represent a widely accepted alternative to primary human hepatocytes due to their stable hepatocyte-like phenotype, robust metabolic competence, formation of bile canaliculi, and suitability for long-term culture under perfusion (Kaden, Graf, et al., 2023; Qiu et al., 2023). Unlike many other hepatic cell lines, differentiated HepaRG cells retain substantial CYP activity and polarization, making them particularly suitable for drug metabolism and toxicity studies in microfluidic environments (Guillouzo

et al., 2007)

Equally important is the incorporation of liver sinusoidal endothelial cells (LSECs), which form the physiological interface between blood flow and hepatocytes. LSECs regulate molecular exchange, maintain hepatocyte differentiation, and mediate shear-dependent signaling within sinusoidal microchannels (Dalsbecker et al., 2022). Their inclusion is therefore essential for recreating the structural and functional characteristics of the hepatic sinusoid described in Section 1.2.

In this thesis, a simplified two-cell-type Liver-on-a-Chip model is established (Figure 3) using differentiated HepaRG hepatocytes and human LSECs on the Dynamic42 platform, following the microphysiological framework demonstrated in prior work (Kaden, Graf, et al., 2023). To ensure reproducibility and long-term stability under perfusion, the endothelial component is based on immortalized human LSECs generated using SV40 large T antigen. SV40-mediated immortalization promotes sustained proliferation through interference with p53- and Rb-dependent cell cycle regulation, while key endothelial properties such as barrier formation and lineage-specific marker expression are retained (Ahuja et al., 2005; Zhu et al., 2018).

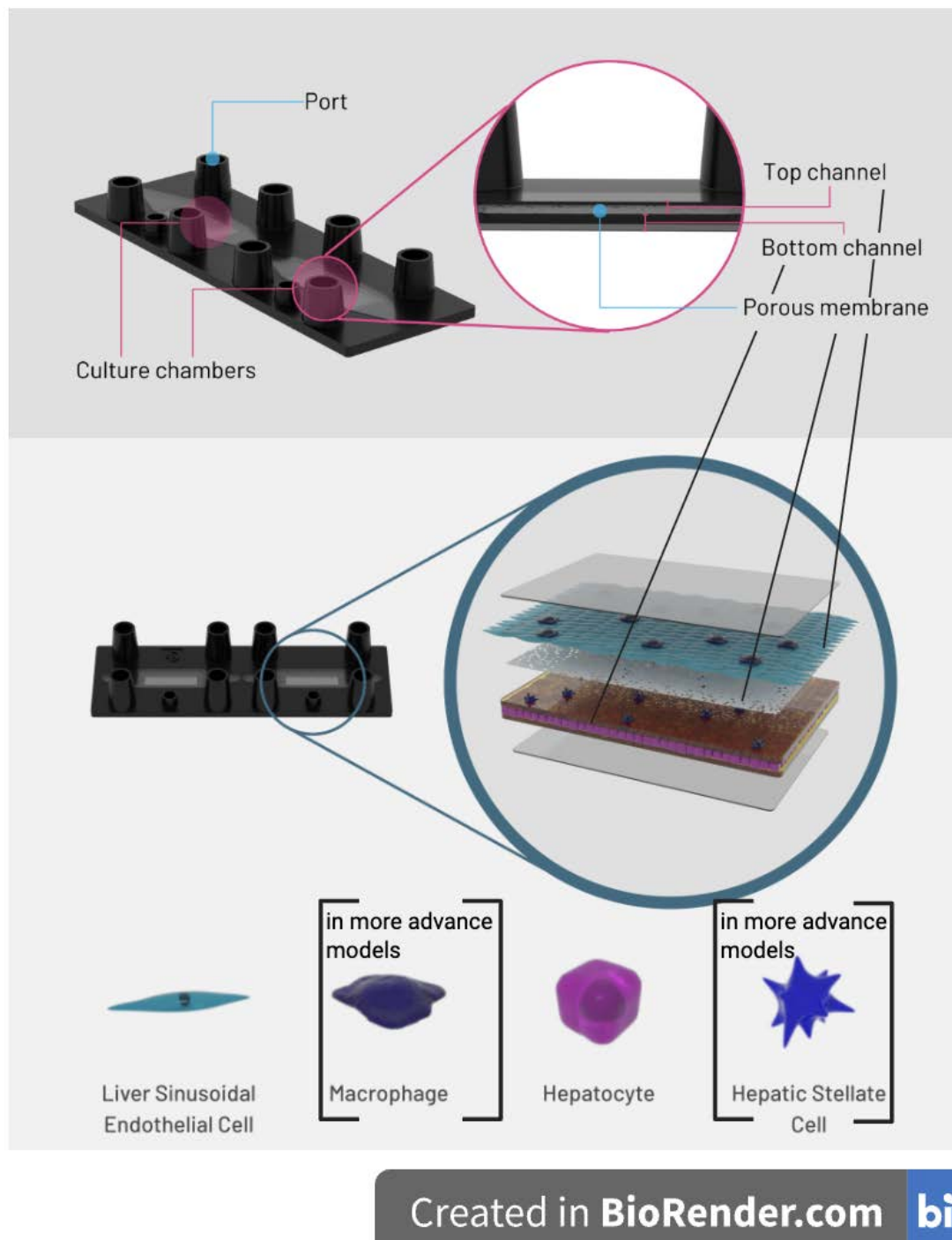


Figure 3: Microphysiological Liver-on-a-Chip platform. The upper panel illustrates the dual-channel architecture of the microfluidic device, consisting of a vascular (top) and a hepatic (bottom) channel separated by a porous membrane that enables controlled molecular exchange while maintaining compartmentalization. The lower panel depicts representative liver-relevant cell types that can be incorporated into the system. In simplified configurations, hepatocytes and liver sinusoidal endothelial cells (LSECs) are cultured on opposite sides of the membrane to recreate a sinusoidal-like interface. More advanced models may additionally include Kupffer cells and hepatic stellate cells to capture immune and fibrotic processes. Illustration created with BioRender.com, based on materials from Dynamic42 GmbH.

The combination of metabolically competent HepaRG hepatocytes with shear-responsive LSECs allows reconstruction of a simplified yet physiologically meaningful sinusoidal interface under controlled flow conditions (Dalsbecker et al., 2022; Qiu et al., 2023). This configuration provides a balanced compromise between biological relevance, technical feasibility, and experimental reproducibility (Bhatia & Ingber, 2014).

Beyond architectural design, functional validation is essential to assess model performance. In this study, lactate dehydrogenase (LDH) release is employed as a marker of membrane integrity and acute hepatocellular injury under dynamic perfusion conditions (Kaden, Graf, et al., 2023; Yang et al., 2022). Quantification of LDH in the culture supernatant enables sensitive detection of drug-induced cytotoxicity and provides a standardized readout for comparing treatment responses within the established microphysiological system (Ding et al., 2017; Kaden, Graf, et al., 2023).

1.6 Long-Term Relevance and Applications

A central advantage of Liver-on-a-Chip (LoC) systems lies in their capacity to maintain hepatic function over extended culture periods. Continuous perfusion, controlled shear stress, and stable microenvironmental conditions preserve metabolic activity, albumin secretion, urea production, and cytochrome P450 (CYP) expression that surpasses conventional 2D cultures and many static 3D systems (Dalsbecker et al., 2022; Kulsharova & Kurmangaliyeva, 2021). This long-term stability enables investigation of chronic drug exposure, delayed toxicity, and cumulative stress responses under conditions that more closely approximate clinical treatment scenarios (Esch et al., 2015; Yang et al., 2022).

Sustained functionality also expands the applicability of LoC models to disease modelling (Qiu et al., 2023). By preserving multicellular interactions and endothelial–parenchymal communication, perfused systems can recreate progressive pathological processes such as steatosis, steatohepatitis, fibrosis, or cholestasis in a controlled environment (Liu et al., 2024; Underhill & Khetani, 2019). Inclusion of non-parenchymal cells, including liver sinusoidal endothelial cells (LSECs), Kupffer cells, and hepatic stellate cells, allows mechanistic investigation of inflammatory signaling, extracellular matrix remodeling, and zonation-dependent metabolic alterations—features that are difficult to capture in static in vitro systems or animal models (Kulsharova & Kurmangaliyeva, 2021; Qiu et al., 2023).

Beyond disease modelling, LoC platforms contribute to precision medicine approaches by enabling integration of patient-derived hepatocytes or induced pluripotent stem cell (iPSC)-derived liver cells (Ingber, 2022). Such personalized systems allow assessment of individual drug responses and genetic susceptibilities in a physiologically relevant

microenvironment. Although still evolving, these applications highlight the broader translational potential of microphysiological liver models (Mugaanyi et al., 2025). Furthermore, Liver-on-a-Chip units can be incorporated into multi-organ platforms to simulate systemic physiology. Coupling hepatic modules with gut, kidney, or immune compartments enables analysis of inter-organ signaling, drug–drug interactions, metabolic activation, and systemic toxicity under dynamic flow conditions (Ingber, 2022; Qiu et al., 2023). Advances in embedded sensing technologies further enhance real-time monitoring of barrier integrity, oxygenation, and inflammatory mediators, strengthening their value for long-term functional analysis (Yang et al., 2022). Collectively, the ability to sustain physiologically relevant liver function under controlled perfusion conditions positions Liver-on-a-Chip systems as powerful tools for chronic toxicity testing, mechanistic disease modelling, and translational pharmacological research (Ingber, 2022; Qiu et al., 2023).

1.7 Aim of This Thesis

The primary aim of this thesis is to establish and technically validate a perfused human Liver-on-a-Chip model as a robust and reproducible microphysiological platform for drug-induced liver injury studies.

Building on previously published microfluidic liver models (Kaden, Graf, et al., 2023), the project focuses on reconstructing a simplified sinusoidal interface under dynamic flow conditions using differentiated HepaRG hepatocytes in combination with endothelial cells cultured on opposite sides of a collagen-coated porous membrane.

To ensure controlled model establishment and minimize experimental risk, the system was initially assembled using HepaRG hepatocytes together with an immortalized human lymphatic endothelial cell (LEC) line on the vascular side. This preparatory phase enabled optimization of chip handling, coating procedures, seeding strategies, and perfusion control under technically accessible and cost-efficient conditions.

Following stabilization of co-culture workflows and baseline performance, the vascular compartment was transitioned to immortalized human liver sinusoidal endothelial cells (LSECs), thereby increasing physiological relevance while maintaining reproducibility and stability under microfluidic perfusion.

A further objective was the implementation and optimization of the complete HepaRG differentiation protocol prior to chip seeding, including proliferation and maturation phases to promote metabolic competence and cellular polarization.

Functional validation of the established model was performed using lactate dehydrogenase (LDH) release as a quantitative marker of drug-induced cytotoxicity under dynamic perfusion. The system was challenged with the clinically relevant antibiotics

levofloxacin and meropenem to assess its capacity to detect treatment-associated stress responses, while staurosporine served as a cytotoxic positive control.

By establishing a technically robust and physiologically informed HepaRG–LSEC Liver-on-a-Chip configuration, this thesis provides a reproducible foundation for future investigations of hepatic drug responses and microenvironment-dependent toxicity in a human-relevant in vitro system.

2 Materials and Methods

2.1 Cell Culture Models

2.1.1 HepaRG Cells

Cell Line

Undifferentiated human HepaRG cells (HPR101) were obtained from Biopredic International (Rennes, France). HepaRG cells are a well-established human hepatic cell line capable of differentiation into hepatocyte-like cells with metabolic functionality comparable to primary hepatocytes (Aninat et al., 2006; Gripon et al., 2002).

Reagents and Media

Table 1: Reagents used for HepaRG cell culture

Reagent	Supplier	Catalog No.
Williams' Medium E (w/o L-glutamine, phenol red)	Sigma-Aldrich	W1878
HyClone FetalClone II Serum	Cytiva	SH30066.03
Penicillin/Streptomycin (100×)	Sigma-Aldrich	P0781
GlutaMAX™ (100×)	Thermo Fisher Scientific	35050038
Insulin	Sigma-Aldrich	I9278
Dexamethasone	Sigma-Aldrich	D4902
Gentamicin	Sigma-Aldrich	G1397
Trypsin/EDTA	Sigma-Aldrich	T3924
PBS	Sigma-Aldrich	20012019
DMSO	Sigma-Aldrich	D2650
Trypan Blue Solution	Sigma-Aldrich	T8154
Primary hepatocyte thawing and plating supplement (CM3000)	Gibco	CM3000
Primary hepatocyte maintenance supplement (CM4000)	Gibco	CM4000

Table 2: Composition of HepaRG culture and differentiation media

Component	Culture Medium	Differentiation Medium
Williams' Medium E	Base	Base
FetalClone II Serum	10%	10%
Penicillin/Streptomycin	1×	1×
GlutaMAX™	1×	1×
Insulin	5 µg/mL	5 µg/mL
Dexamethasone	50 µM	50 µM
Gentamicin	80 µg/mL	80 µg/mL
DMSO	—	2%

Thawing and Initial Seeding

Cryopreserved HepaRG cells were rapidly thawed in a 37°C water bath until only a small ice crystal remained. Cells were transferred into pre-warmed culture medium and gently resuspended. Cell number and viability were determined using trypan blue exclusion. Cells were seeded into tissue culture-treated flasks (T25 or T75) and incubated under standard conditions (37°C, 5% CO₂). Medium was replaced approximately 6 hours after seeding to remove residual cryoprotectant and subsequently every 2–3 days.

Routine Culture and Passaging

Cells were cultured until reaching confluence within approximately two weeks. For passaging, cells were washed once with PBS and detached using Trypsin/EDTA for 3–5 minutes at 37°C. Enzymatic detachment was stopped by addition of culture medium, and cells were carefully resuspended to obtain a single-cell suspension. Cell number and viability were assessed prior to reseeding.

Cells were routinely passaged approximately once per week and seeded at defined densities depending on the culture format (Table 3).

Table 3: Seeding densities and medium volumes for HepaRG cultures

Culture Format	Cell Number	Medium Volume
T25 flask	0.5×10^6 cells/flask	5 mL
T75 flask	2×10^6 cells/flask	10–15 mL

Differentiation of HepaRG Cells

For differentiation, HepaRG cells were cultured until reaching full confluence. Medium was then replaced with differentiation medium containing 2% DMSO. During the first 2–3 days, a gradual transition was performed using a 1:1 mixture of culture and differentiation medium, followed by full differentiation medium.

Cells were differentiated for a total period of two weeks, with medium changes every 2–3 days. Differentiated HepaRG cells displayed characteristic hepatocyte-like morphology and liver-specific functionality as previously described (Aninat et al., 2006).

Cryopreservation

For cryopreservation, cells were harvested as described for passaging and centrifuged at $300 \times g$ for 5 minutes. The cell pellet was resuspended in freezing medium consisting of culture medium supplemented with 10% DMSO at a density of $1\text{--}2 \times 10^6$ cells/mL. Aliquots of 1 mL were transferred into cryovials and stored at -80°C for several days before transfer to liquid nitrogen for long-term storage.

2.1.2 Lymphatic Endothelial Cells (LECs)

Cell Line

Lymphatic endothelial cells (LECs) were kindly provided by Bettina Grasl (Medical University of Vienna). The cells originate from telomerase-immortalized endothelial cells obtained from Georg Krupitza (AKH Vienna) and were previously described by (Schoppmann et al., 2004). These cells were used during the initial optimization phase of the microfluidic system.

Reagents and Media

Table 4: Reagents used for LEC culture

Reagent	Supplier	Catalog No.
EBM-2 Basal Medium	Lonza	CC-3202
Penicillin/Streptomycin (100×)	Sigma-Aldrich	P0781
GlutaMAX™ (100×)	Thermo Fisher Scientific	35050038
Trypsin/EDTA	Sigma-Aldrich	T3924
PBS	Sigma-Aldrich	20012019
DMSO	Sigma-Aldrich	D2650
Trypan Blue Solution	Sigma-Aldrich	T8154
Collagen A Type I (1 mg/mL)	Pan Biotech	P06-20030

Table 5: Composition of LEC culture medium (EGM-2 based)

Component	Final Concentration
EBM-2 Basal Medium	Base
Fetal calf serum (FCS)	5%
Hydrocortisone	As provided
rhFGF-B	As provided
VEGF	As provided
R3 IGF-1	As provided
Ascorbic acid	As provided
rhEGF	As provided
Gentamicin sulfate (GA-1000)	As provided

LECs were cultured in complete EBM-2 medium supplemented with growth factors according to the manufacturer’s instructions (EGM-2 kit, Lonza).

Collagen Coating of Culture Flasks

For optimal cell attachment, culture flasks were coated with collagen type I. Collagen A was diluted in PBS to a final concentration of 0.05 mg/mL (1:20 dilution from stock solution). Approximately 3 mL of collagen solution were added to T25 flasks to fully cover the growth surface and incubated at 37°C for at least 1 hour or overnight. Excess collagen was removed, and flasks were washed twice with PBS prior to use. Coated flasks were used immediately or stored at 4°C for short-term use.

Thawing and Initial Culture

Cryopreserved LECs were rapidly thawed in a 37°C water bath until only a small ice crystal remained. Cells were disinfected with 70% ethanol and transferred into a sterile 50 mL conical tube containing pre-warmed culture medium. The cryovial was rinsed with additional medium to maximize cell recovery. Cell number and viability were determined using trypan blue exclusion.

Cells were seeded into collagen-coated T25 flasks and incubated under standard conditions (37°C, 5% CO₂). Medium was replaced approximately 6 hours after seeding to remove residual cryoprotectant.

Routine Culture and Passaging

LECs were maintained under standard cell culture conditions and passaged one to two times per week at a split ratio of approximately 1:5 to 1:10. For passaging, cells were washed once with PBS and detached using Trypsin/EDTA (approximately 1 mL per T25 flask) for 3–5 minutes at 37°C. Enzymatic activity was stopped by addition of culture medium, and cells were gently resuspended to obtain a homogeneous single-cell suspension.

Cells were counted and reseeded into freshly collagen-coated culture flasks. Medium was changed every 2–3 days.

Cryopreservation

For cryopreservation, cells were harvested as described for passaging and centrifuged at $300 \times g$ for 5 minutes. The cell pellet was resuspended in freezing medium consisting of complete EBM-2 medium supplemented with 10% DMSO at a density of $0.5\text{--}1 \times 10^6$ cells/mL. Cell suspensions were aliquoted into cryovials and stored at -80°C for several days before transfer to liquid nitrogen for long-term storage.

2.1.3 Liver Sinusoidal Endothelial Cells (LSECs)

Cell Line

Immortalized human liver sinusoidal endothelial cells (LSECs) (Innoprot, P10652-IM) were used in the final experimental setup. In contrast to LECs, LSECs were employed for the fully established Liver-on-a-Chip model to better recapitulate the physiological liver sinusoidal endothelium. LSECs represent a specialized endothelial cell population of the hepatic sinusoid with distinct functional properties and have been previously characterized (Milani et al., 2024).

Reagents and Media

Table 6: Reagents used for LSEC culture

Reagent	Supplier	Catalog No.
ECM Medium Kit	Innoprot	P60104
Fetal bovine serum (FBS) supplement	Innoprot	161625
Endothelial cell growth supplement (100×)	Innoprot	26024
Penicillin/Streptomycin solution (100×)	Innoprot	26B05
Trypsin/EDTA	Sigma-Aldrich	T3924
PBS	Sigma-Aldrich	20012019
DMSO	Sigma-Aldrich	D2650
Trypan Blue Solution	Sigma-Aldrich	T8154
Collagen A Type I (1 mg/mL)	Pan Biotech	P06-20030
G418 (Geneticin, 100 mg/mL solution)	InvivoGen	ant-gn-1

Table 7: Composition of LSEC culture medium

Component	Final Composition
ECM basal medium	Base
FBS supplement	5% (25 mL per 500 mL)
Endothelial cell growth supplement	1× (5 mL per 500 mL)
Penicillin/Streptomycin	1× (5 mL per 500 mL)

LSECs were cultured in complete ECM medium supplemented according to the manufacturer's instructions.

Collagen Coating of Culture Flasks

To ensure proper cell adhesion, culture flasks were coated with collagen type I. Collagen A was diluted in PBS to a final concentration of 0.05 mg/mL (1:20 dilution from stock solution). Approximately 3 mL of collagen solution were added to T25 flasks to fully cover the growth surface and incubated at 37°C for at least 1 hour or overnight. After incubation, flasks were washed twice with PBS and used immediately or stored short-term.

Thawing and Initial Culture

Cryopreserved LSECs were rapidly thawed in a 37°C water bath until only small ice crystals remained. After disinfection of the vial with 70% ethanol, cells were transferred into a sterile 50 mL conical tube containing pre-warmed culture medium. The vial was rinsed with additional medium to maximize cell recovery.

An aliquot of the cell suspension was used to determine cell number and viability via trypan blue exclusion. Cells were subsequently seeded into collagen-coated T25 flasks and incubated at 37°C and 5% CO₂. Medium was replaced approximately 24 hours after seeding.

Routine Culture and Passaging

LSECs were maintained under standard cell culture conditions and passaged one to two times per week at a split ratio of approximately 1:2 to 1:3. Medium was changed every 2–3 days.

For passaging, cells were washed once with PBS and detached using Trypsin/EDTA (approximately 1 mL per T25 flask) for 3–5 minutes at 37°C. Enzymatic activity was stopped by addition of culture medium, and cells were gently resuspended to obtain a homogeneous suspension. Cell number and viability were determined prior to reseeding into freshly collagen-coated flasks.

To maintain selection pressure, cells were intermittently cultured in the presence of G418 (Geneticin), applied after every 3–4 passages.

Cryopreservation

For cryopreservation, cells were harvested as described above and centrifuged at 300 × g for 5 minutes. The cell pellet was resuspended in freezing medium consisting of complete ECM medium supplemented with 10% DMSO at a density of 0.5–1 × 10⁶ cells/mL.

Aliquots of 1 mL were transferred into cryovials, gradually cooled at -80°C, and subsequently stored in liquid nitrogen for long-term preservation.

2.2 Microfluidic Liver-on-a-Chip Model

2.2.1 Chip Platform

Microfluidic experiments were performed using the DynamicOrgan® 2-Channel system (Dynamic42 GmbH) equipped with BC002 biochips. Experimental procedures were adapted from manufacturer protocols (Dynamic42 GmbH, 2024a, 2024b). Each biochip

consists of two parallel microchannels separated by a porous polyethylene terephthalate (PET) membrane (pore size: $8\ \mu\text{m}$; pore density: 1×10^5 pores/ cm^2), enabling compartmentalized co-culture of endothelial and hepatic cells.

Table 8: Materials used for microfluidic experiments

Material	Supplier
DynamicOrgan Developer Kit (small)	Dynamic42 GmbH
Biochips (BC002)	Dynamic42 GmbH
2-stop tubing	Dynamic42 GmbH
Luer adapters and plugs	Dynamic42 GmbH
Reservoirs	Dynamic42 GmbH
Peristaltic pump	Dynamic42 GmbH

2.2.2 Chip Sterilization and Coating

Prior to cell seeding, biochips were sterilized by complete immersion in 70% ethanol for 30 minutes under sterile conditions. All channels were carefully filled to avoid air bubble formation. After sterilization, ethanol was removed and chips were extensively washed with sterile distilled water to eliminate residual ethanol.

To promote cell adhesion, both channels were coated with collagen type I (0.05 mg/mL in PBS). Channels were filled with collagen solution (approximately 250 μL per channel) and incubated at 37°C for at least 1 hour or overnight. Before cell seeding, channels were washed with PBS to remove excess collagen.

2.2.3 Cell Seeding

Endothelial Cell Seeding

Endothelial cells (LECs during optimization, LSECs for final experiments) were seeded into the upper (vascular) channel. Cells were prepared as single-cell suspensions and seeded at a density of $0.5\text{--}1.0 \times 10^5$ cells per channel in 200 μL of respective endothelial medium (EBM-2 for LECs, ECM medium for LSECs).

After seeding, channels were closed and chips were incubated under static conditions (37°C , 5% CO_2) to allow cell attachment and monolayer formation. Endothelial cells typically reached confluence within approximately 3 days.

HepaRG Seeding

After establishment of a confluent endothelial layer, differentiated HepaRG cells were seeded into the lower (hepatic) channel. Cells were detached, counted, and resuspended in HepaRG WME seeding medium (see Section 2.2.5).

Cells were seeded at a density of 3.0×10^5 cells per channel in 150 μL . Following seeding, the chip was inverted to facilitate cell attachment to the underside of the membrane and incubated overnight under standard conditions.

2.2.4 Medium Exchange and Culture Conditions

During the static culture phase, medium in both channels was exchanged daily. For endothelial cells, EBM-2 or ECM medium was used depending on the cell type. HepaRG cells were maintained in WME seeding medium.

Care was taken to avoid air bubble formation during medium exchange by slowly pipetting medium into the channel inlets.

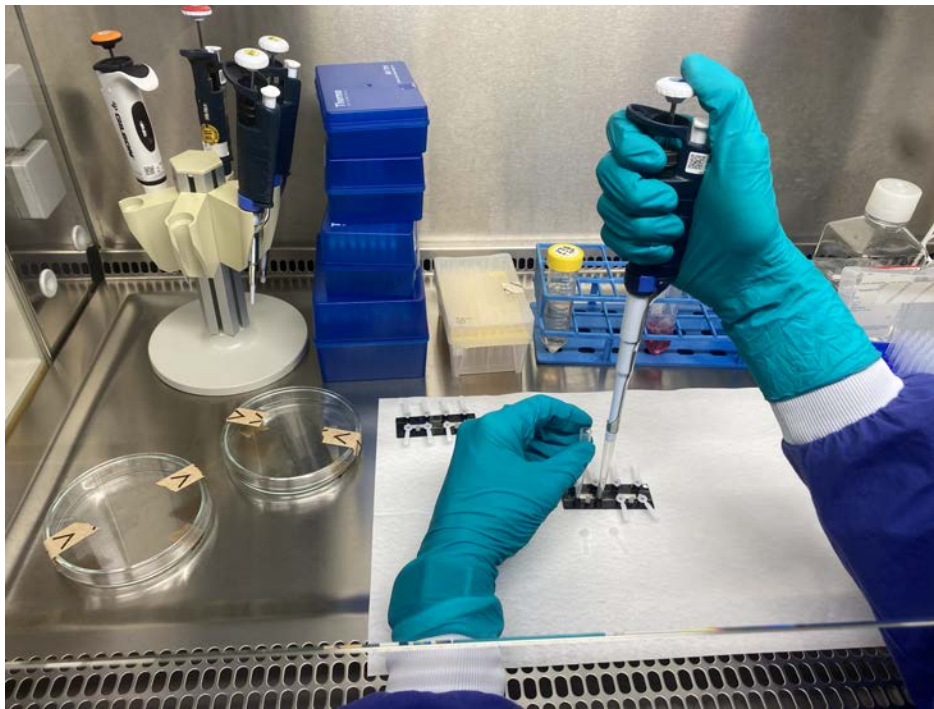


Figure 4: Medium exchange on chip under sterile conditions. Fresh medium was introduced into the right inlet of the chamber using a pipette, while the old medium was simultaneously removed from the opposite outlet using a 1000 μL pipette tip. To prevent uncontrolled leakage, the pipette tip was sealed with a finger after the medium had entered the tip.

2.2.5 HepaRG Culture Media for On-Chip Experiments

For on-chip experiments, two different Williams' Medium E (WME)-based media were used for HepaRG cells depending on the experimental phase: a seeding medium during the static culture phase and a perfusion medium during flow conditions.

The WME seeding medium was used for cell seeding to support initial cell attachment and stabilization on-chip. Upon initiation of perfusion, the medium was switched to a WME perfusion medium optimized for flow conditions.

Both media were prepared freshly and supplemented as described below.

Table 9: Composition of WME-based seeding and perfusion media for on-chip HepaRG culture

Component	Seeding Medium	Perfusion Medium
Williams' Medium E	Base	Base
FetalClone II Serum	5%	10%
Penicillin/Streptomycin	1×	1×
GlutaMAX™	1×	1×
Insulin	5 µg/mL	5 µg/mL
Dexamethasone	5 µM	5 µM
DMSO	–	0.1%
CM3000 (Gibco)	4%	–
CM4000 (Gibco)	–	3.6%

2.2.6 Perfusion Setup and Pre-conditioning

After establishment of stable co-cultures, perfusion was initiated. A 24-hour pre-perfusion phase was conducted to allow cellular adaptation to flow conditions.

Perfusion was applied exclusively to the vascular (upper) channel at a flow rate of 25 µL/min using a peristaltic pump. The flow direction was kept constant throughout the experiment.

The perfusion system (tubing, reservoirs, connectors) was assembled under sterile conditions and pre-filled with medium to avoid introduction of air bubbles. The pump system was equilibrated in the incubator prior to connection to the chips.

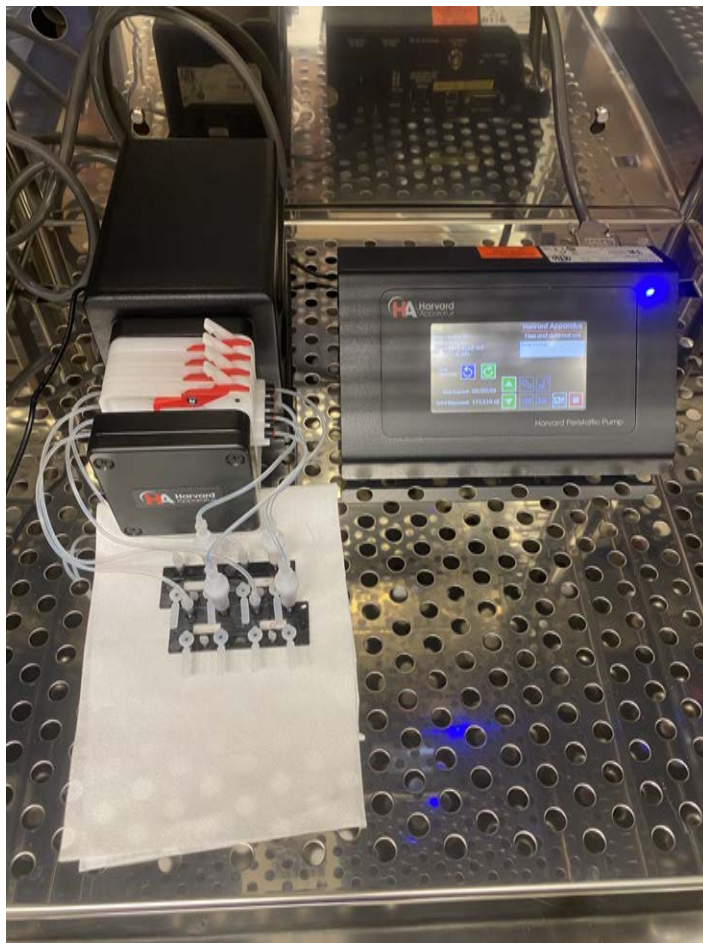


Figure 5: Experimental setup of the Liver-on-a-Chip system. The DynamicOrgan® platform (Dynamic42 GmbH), including peristaltic pump, tubing, reservoirs, and biochips, was assembled and operated under standard cell culture conditions inside a humidified incubator (37°C, 5% CO₂).

2.2.7 Drug Treatment under Perfusion

Following pre-perfusion, drug treatments were initiated under continuous flow conditions. Media in reservoirs were supplemented with respective compounds and replaced daily.

Table 10: Drug treatments and concentrations

Compound	Supplier	Catalog No.	Final Concentration
Staurosporine (positive control)	Sigma-Aldrich	S4400	10 μ M
Levofloxacin	Sigma-Aldrich	28266	10 μ M
Meropenem	AptaMedica	—	100 μ M

Levofloxacin and meropenem concentrations were selected based on clinically relevant

plasma concentrations ($C_{\max}$).

Stock solutions of levofloxacin (Sigma-Aldrich, 28266) and meropenem (AptaMedica) were prepared by dissolving the respective powders in sterile water. Staurosporine stock solution was prepared in dimethyl sulfoxide (DMSO).

Working concentrations were obtained by diluting the respective stock solutions in culture medium immediately prior to use.

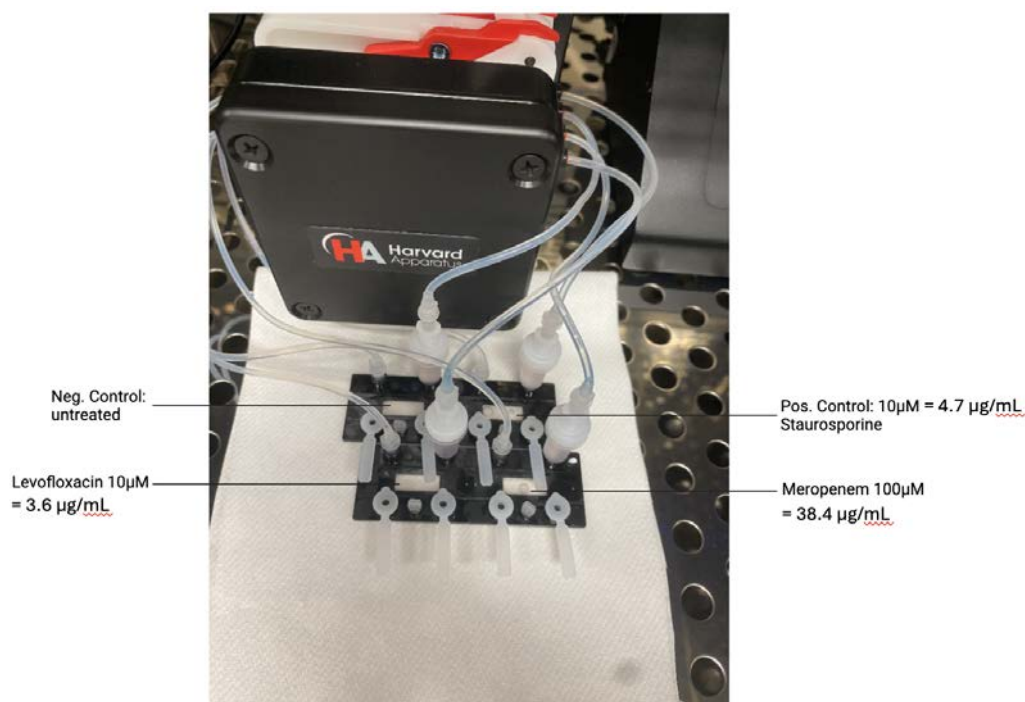


Figure 6: Drug treatment setup under perfusion conditions. Reservoirs were supplied with medium containing the respective compounds, including negative control, positive control (staurosporine), levofloxacin, and meropenem, enabling parallel treatment of individual chip chambers.

2.2.8 Microscopy and Image Acquisition

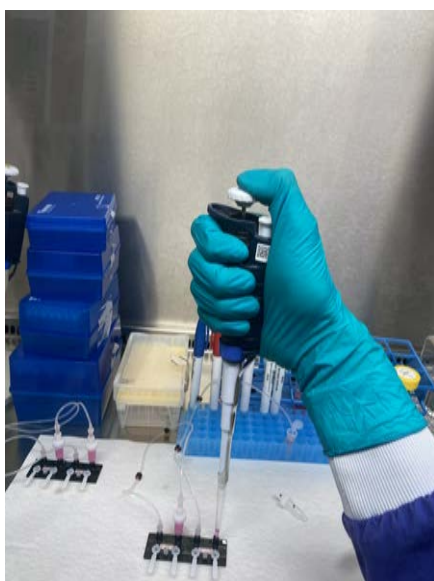
Cell morphology and confluence within the microfluidic chips were monitored daily using an inverted microscope (Olympus, model EP50 camera system). Brightfield images were acquired from both the vascular (endothelial) and hepatic compartments throughout the course of the experiments.

Imaging was performed under sterile conditions where possible, and identical acquisition settings were maintained across all experimental groups to ensure comparability. Representative images were recorded for each treatment condition every 24 hours during perfusion.

2.2.9 Sampling and Medium Exchange

Medium was replaced daily in the reservoirs during perfusion experiments. Supernatants were collected daily from both vascular and hepatic compartments and stored at -80°C for downstream analysis.

To collect samples, perfusion was temporarily stopped, and medium from reservoirs and chip outlets was carefully retrieved while avoiding introduction of air bubbles. Fresh medium containing the respective treatment was subsequently added, and perfusion was resumed.



(a) Vascular (endothelial) compartment



(b) Hepatic compartment

Figure 7: Collection of supernatants from the chip during perfusion experiments. Perfusion was temporarily paused and medium was flushed through the outlet tubing and collected from both vascular and hepatic compartments while avoiding air bubble formation. Fresh medium containing the respective treatment was subsequently added before perfusion was resumed.

2.2.10 Cleaning and Reuse of the Perfusion System

After completion of experiments, perfusion systems were cleaned by flushing tubing and reservoirs with 70% ethanol followed by extensive washing with distilled water. Components were air-dried and autoclaved prior to reuse.

Reusable components were handled carefully to prevent contamination and ensure consistent flow conditions in subsequent experiments.

2.3 LDH Cytotoxicity Assay

To assess cell damage during the microfluidic experiments, lactate dehydrogenase (LDH) release was quantified in daily collected supernatants from both the hepatic (HepaRG) and vascular (endothelial) compartments. LDH is released upon loss of membrane integrity and therefore serves as a marker of cytotoxicity.

Cell damage was determined using a commercially available LDH assay kit (ab65393, Abcam) according to the manufacturer’s protocol.

Table 11: Materials used for LDH assay

Material	Specification
LDH assay kit	ab65393 (Abcam)
96-well plate	Greiner Bio-One, 655001
Microplate reader	BioTek Synergy H1

Supernatants were centrifuged ($600 \times g$, 10 min) to remove cellular debris prior to analysis. Samples (10 μ L) were incubated with the reaction mixture for 30 minutes at room temperature. Absorbance was measured at 450 nm with background correction at 650 nm. Background values (650 nm) were subtracted from the corresponding 450 nm measurements. In addition, blank controls consisting of cell culture medium (HepaRG medium and endothelial cell medium) were included and subtracted from all measurements.

Cytotoxicity was calculated as:

$$\text{Cytotoxicity (\%)} = \frac{\text{Sample} - \text{Low control}}{\text{High control} - \text{Low control}} \times 100$$

The high control was defined as the maximum LDH release obtained from lysed cells. For this purpose, HepaRG cells and endothelial cells were lysed separately (300,000 cells each), and their respective absorbance values were used as cell type-specific high controls.

The low control corresponded to the baseline LDH release and was defined as the untreated negative control for each respective day and cell type (HepaRG and endothelial cells).

Data visualization and graph generation were performed using GraphPad Prism version 11.0.2 (GraphPad Software, Boston, USA). Data are presented as mean $\pm$ standard deviation (SD) from three independent experiments ($n = 3$). Due to the limited number of independent experiments, no inferential statistical analyses were performed, as the sample size was insufficient for meaningful statistical testing. Therefore, the results

were evaluated descriptively and interpreted in conjunction with the morphological assessment.

2.4 Experimental Timeline Overview

To provide an overview of the experimental workflow, a summarized timeline of all major steps is shown in Figure 8. The timeline illustrates the chronological sequence of cell culture, differentiation, chip preparation, seeding procedures, and perfusion-based experiments.

Particular emphasis is placed on the preparatory phase prior to on-chip experiments, which represents a substantial portion of the total experimental duration. This includes the expansion and differentiation of HepaRG cells as well as the preparation of endothelial cells.

In addition, the timeline highlights key experimental phases such as static culture, initiation of perfusion, daily redosing of compounds, and repeated sampling of supernatants for downstream analysis.

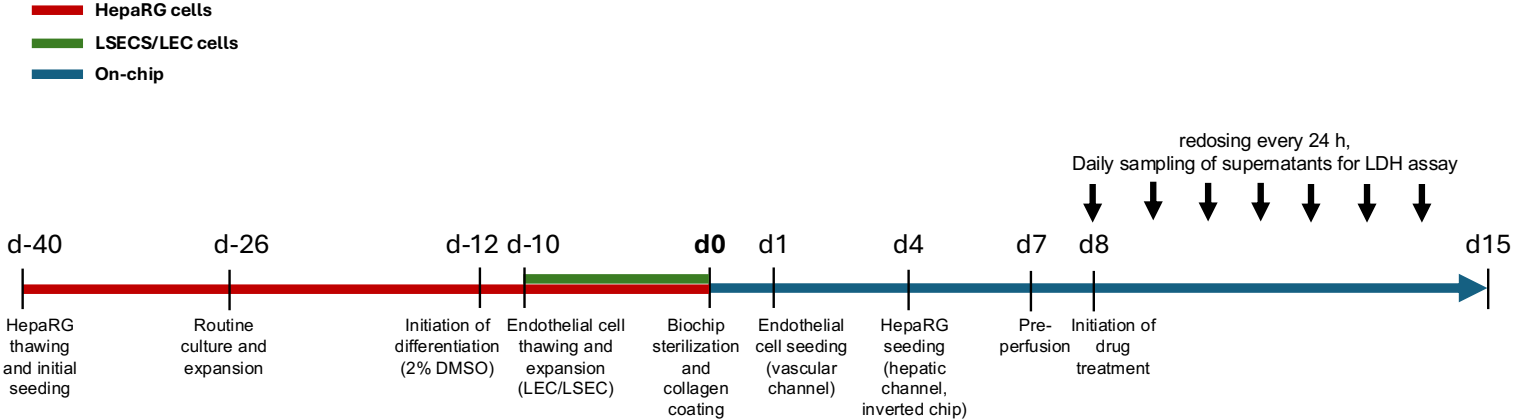


Figure 8: Experimental timeline of the microfluidic Liver-on-a-Chip workflow. The schematic provides an overview of all major steps, from initial HepaRG thawing and differentiation to the final on-chip experiment, including endothelial cell preparation, chip seeding, perfusion, drug treatment, and daily sampling.

3 Results

This chapter presents the results obtained during the establishment and characterization of the microfluidic Liver-on-a-Chip model. The experimental workflow included the differentiation of HepaRG cells, the optimization of endothelial cell culture conditions, the establishment of perfused co-cultures, and the assessment of drug-induced cytotoxicity using LDH release measurements. Results are presented in the chronological order of model development and validation.

3.1 Differentiation and Morphological Characterization of HepaRG Cells

Prior to on-chip experiments, HepaRG cells were differentiated for 14 days in the presence of 2% DMSO. Morphological changes were monitored by brightfield microscopy to assess successful differentiation into a hepatocyte-like phenotype. Undifferentiated HepaRG cells typically exhibit a homogeneous epithelial morphology, whereas differentiated cultures develop characteristic hepatocyte-like colonies with polygonal cells, distinct cell borders, increased cytoplasmic granularity, and bile canaliculi-like structures.

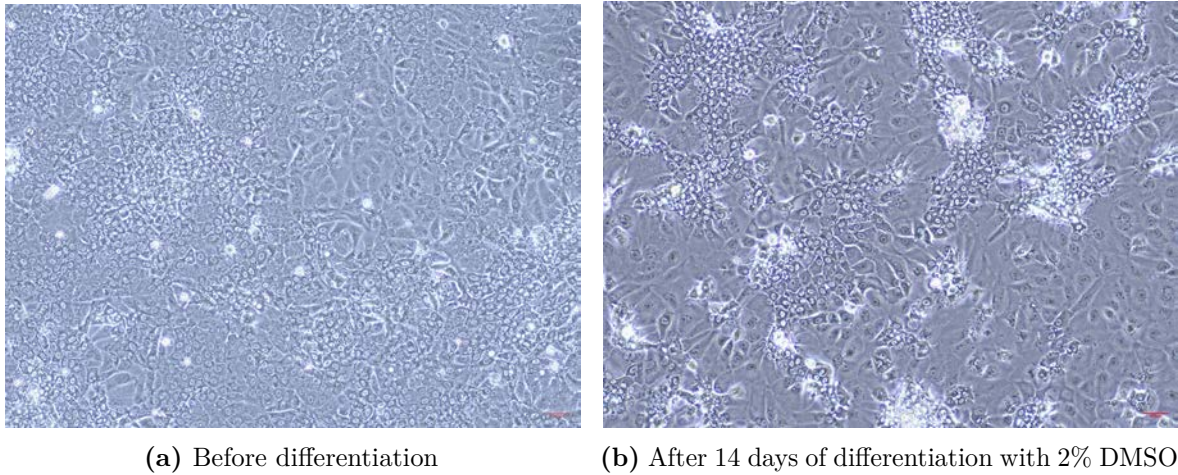


Figure 9: Morphological differentiation of HepaRG cells. Brightfield images of HepaRG cultures before (A) and after (B) differentiation. Prior to DMSO treatment, cells displayed a less organized morphology with a predominance of proliferative cell populations. Following 14 days of differentiation in the presence of 2% DMSO, cultures exhibited the characteristic heterogeneous morphology of differentiated HepaRG cells, including hepatocyte-like colonies surrounded by biliary-like cell populations. Scale bar = 50 μm .

As shown in Figure 9, HepaRG cultures displayed pronounced morphological changes during differentiation. Prior to DMSO treatment, cells formed a relatively uniform

and proliferative monolayer. Following differentiation, distinct hepatocyte-like colonies became apparent and were surrounded by smaller biliary-like cells, consistent with the characteristic morphology of differentiated HepaRG cultures (Cerec et al., 2007; Mayati et al., 2018).

These morphological features were consistently observed in differentiated HepaRG cultures prior to their use in on-chip experiments (Cerec et al., 2007; Mayati et al., 2018).

3.2 Establishment and Characterization of Endothelial Cell Cultures

Endothelial cell cultures were established to generate the vascular compartment of the Liver-on-a-Chip model. Initial optimization experiments were performed using lymphatic endothelial cells (LECs), followed by the implementation of liver sinusoidal endothelial cells (LSECs) for the final experimental setup.

3.2.1 Optimization Experiments Using Lymphatic Endothelial Cells (LECs)

LECs were initially used during the optimization phase of the Liver-on-a-Chip workflow. Prior to chip seeding, cells exhibited a typical endothelial-like morphology characterized by elongated and spindle-shaped cell bodies (Figure 10).

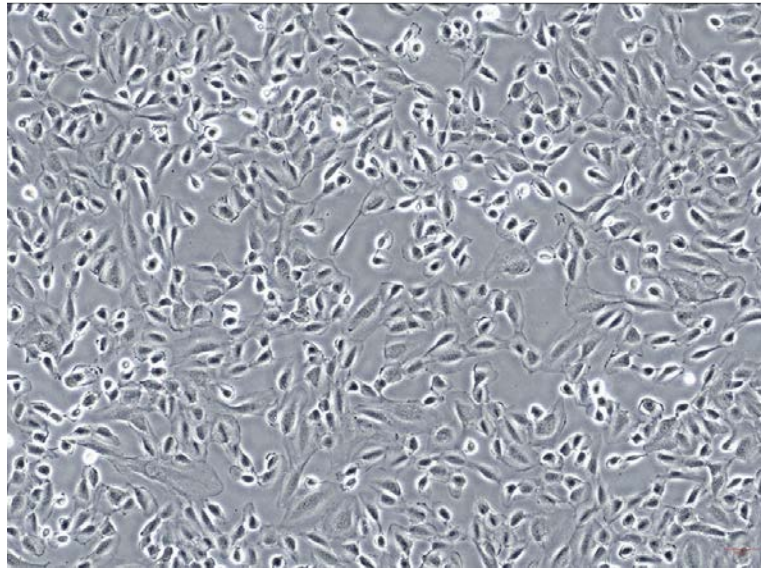


Figure 10: Morphology of lymphatic endothelial cells (LECs) in conventional culture. Brightfield microscopy image showing LECs prior to chip seeding. Cells displayed an elongated endothelial-like morphology and formed adherent cultures suitable for subsequent microfluidic experiments. Scale bar = 100 μm .

LEC cultures showed stable growth characteristics and could be expanded reproducibly for repeated optimization experiments. These cells were therefore used to establish and optimize chip handling procedures, including cell seeding, medium exchange, perfusion setup, and sample collection before implementation of the final HepaRG–LSEC co-culture model.

3.2.2 Establishment of Liver Sinusoidal Endothelial Cell (LSEC) Cultures

Following the optimization phase using LECs, liver sinusoidal endothelial cells (LSECs) were introduced as the endothelial component of the final Liver-on-a-Chip model.

LSEC cultures displayed an adherent endothelial morphology and could be expanded successfully under standard culture conditions. Cells formed homogeneous monolayers and exhibited the elongated and polygonal morphology characteristic of endothelial cell cultures (Figure 11).

The successful cultivation of LSECs enabled their subsequent use in all HepaRG–LSEC co-culture experiments performed on-chip.

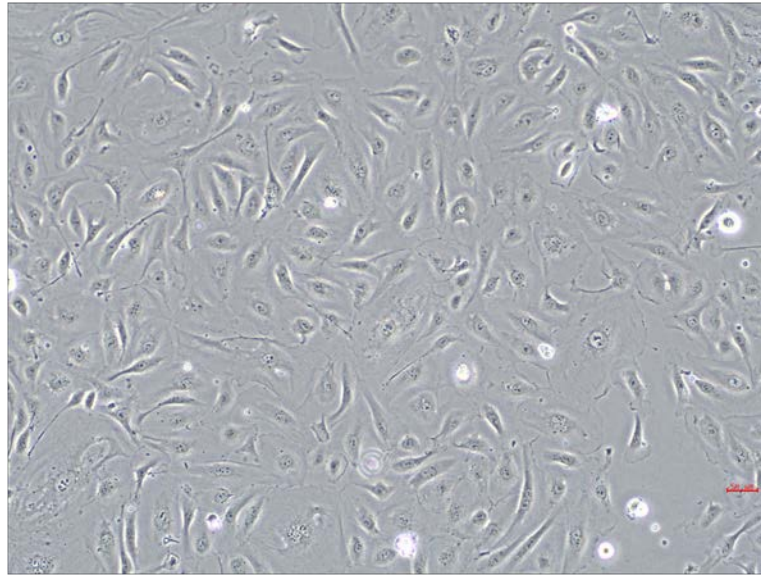


Figure 11: Morphology of liver sinusoidal endothelial cells (LSECs) in conventional culture. Brightfield microscopy image of LSECs prior to chip seeding. Cells formed adherent cultures and displayed the elongated and polygonal morphology characteristic of endothelial cells. Scale bar = 50 μm .

3.3 Initial Optimization of the Liver-on-a-Chip Workflow Using HepaRG–LEC Co-Cultures

Initial optimization experiments were performed using HepaRG cells and lymphatic endothelial cells (LECs) to establish the microfluidic workflow prior to implementation of the final HepaRG–LSEC model. These experiments were used to evaluate cell seeding, attachment, co-culture stability, perfusion, and sampling procedures under experimental conditions.

3.3.1 Cell Seeding and On-Chip Attachment

Following seeding into the microfluidic chip, both cell types successfully attached to their respective compartments. LECs seeded into the vascular channel formed adherent cultures on the membrane surface, while differentiated HepaRG cells attached to the opposite side of the membrane following chip inversion.

Microscopic examination confirmed successful cell attachment and survival of both cell populations prior to initiation of perfusion (Figure 12). These observations demonstrated the feasibility of establishing compartmentalized HepaRG–LEC co-cultures within the microfluidic platform.

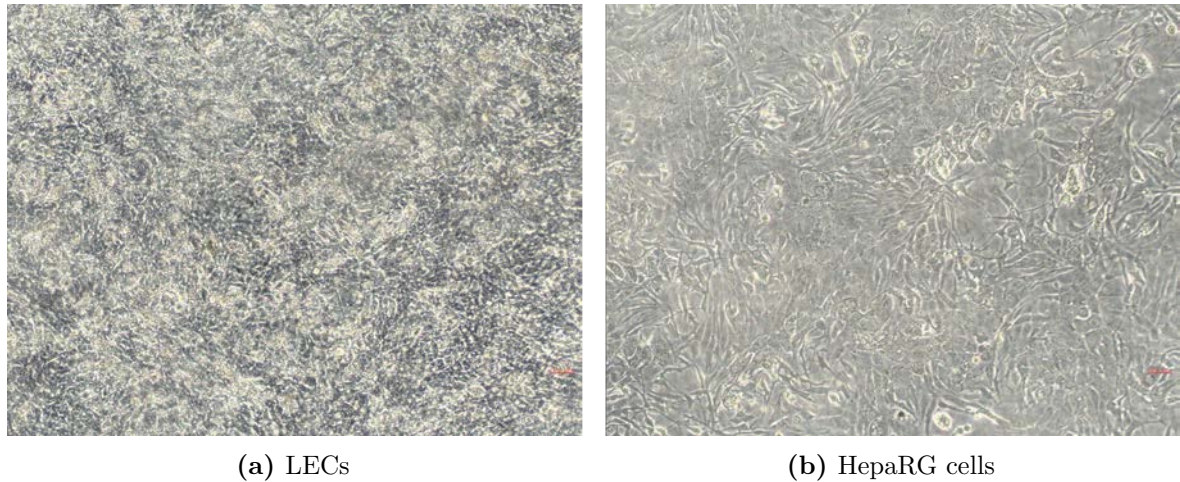


Figure 12: Cell attachment following seeding of HepaRG-LEC co-cultures on-chip. Brightfield images obtained prior to initiation of perfusion. LECs were seeded into the vascular compartment (A), whereas differentiated HepaRG cells were seeded into the hepatic compartment (B). Both cell types successfully attached to the chip membrane and remained adherent during the static stabilization phase. Scale bars = 50 μm .

3.3.2 Response of HepaRG-LEC Co-Cultures to Drug Treatment

Following successful establishment of HepaRG-LEC co-cultures, the response of the model to drug treatment was evaluated under perfusion conditions.

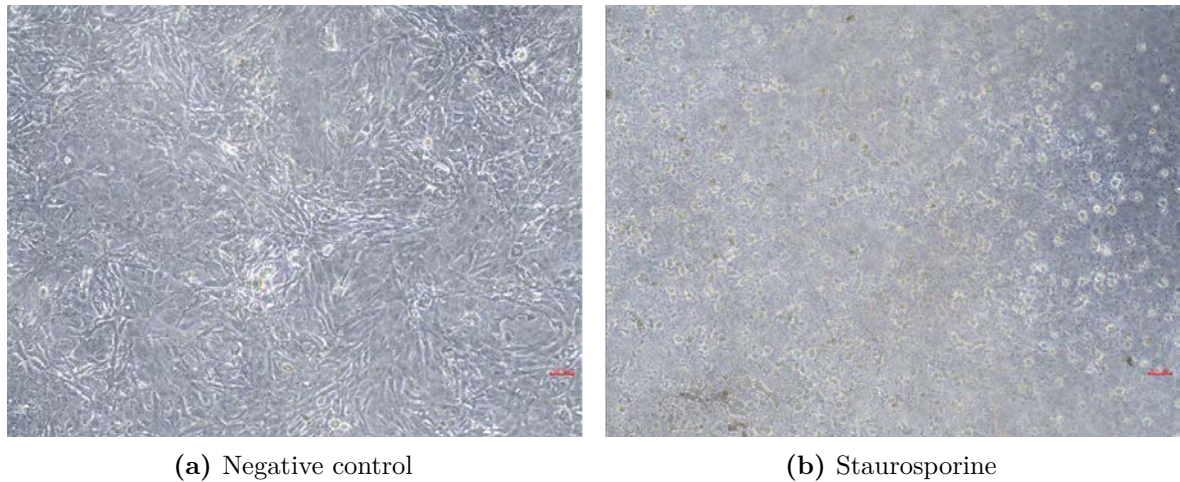


Figure 13: Morphological response of HepaRG-LEC co-cultures to drug treatment. Representative brightfield images of the HepaRG compartment acquired after three days of treatment under perfusion conditions. Untreated control cultures (A) maintained an intact cellular morphology, whereas exposure to staurosporine (B) resulted in extensive cell rounding, loss of adhesion, and marked deterioration of culture integrity. Scale bars = 50 μm .

Clear morphological differences between treatment groups were observed after three days of exposure (Figure 13).

Untreated control cultures maintained a dense and adherent cellular architecture throughout the experiment. In contrast, cultures exposed to the positive control compound staurosporine exhibited extensive morphological deterioration, including cell rounding, detachment, and a substantial reduction in overall culture integrity.

These observations demonstrated that the microfluidic co-culture system was capable of detecting treatment-induced cytotoxic effects and confirmed the suitability of the platform for subsequent HepaRG–LSEC experiments.

3.4 Establishment of a Perfused HepaRG–LSEC Liver-on-a-Chip Model

Following optimization of the workflow using HepaRG–LEC co-cultures, liver sinusoidal endothelial cells (LSECs) were implemented to establish the final Liver-on-a-Chip model. Successful formation of compartmentalized co-cultures and their maintenance under perfusion conditions were assessed by daily microscopic examination.

3.4.1 Formation of Endothelial and Hepatic Compartments

Following establishment of the final HepaRG–LSEC co-culture model, both cell types successfully attached to their respective compartments within the microfluidic chip. Microscopic examination performed prior to initiation of compound treatment confirmed the presence of viable and adherent cell populations on both sides of the porous membrane.

LSECs formed a dense endothelial layer within the vascular compartment and displayed the elongated morphology characteristic of endothelial cells. In parallel, differentiated HepaRG cells remained adherent within the hepatic compartment and retained their characteristic morphology (Figure 14).

These observations confirmed successful establishment of the compartmentalized HepaRG–LSEC Liver-on-a-Chip model and provided the basis for subsequent perfusion and drug treatment experiments.

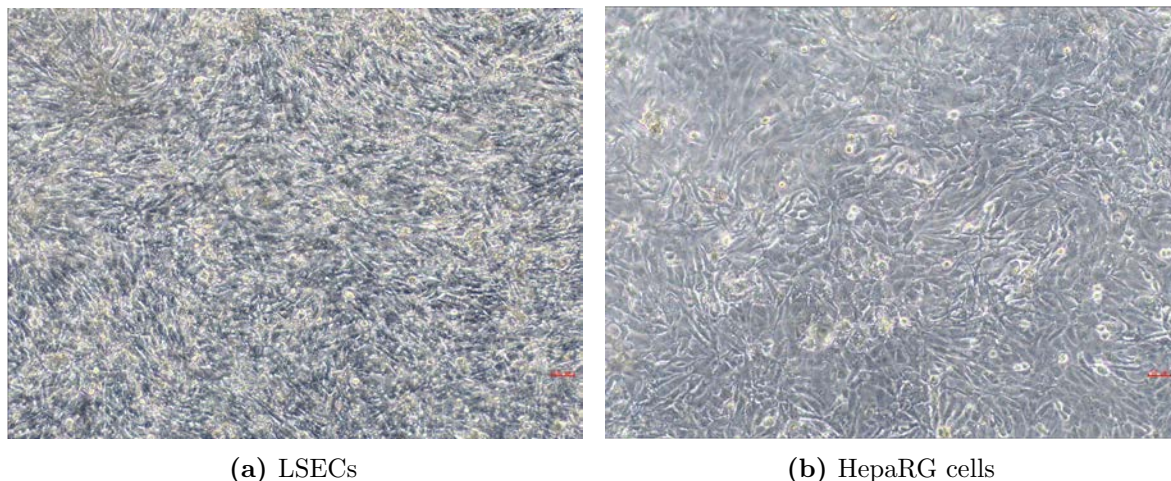


Figure 14: Formation of endothelial and hepatic compartments in the final HepaRG–LSEC Liver-on-a-Chip model. Brightfield images acquired immediately prior to initiation of compound treatment. LSECs formed an adherent endothelial layer within the vascular compartment (A), whereas differentiated HepaRG cells remained attached within the hepatic compartment (B). Both cell populations displayed viable morphology and were maintained successfully under on-chip culture conditions. Scale bars = 50 μm .

3.4.2 Maintenance of Co-Culture Morphology During Perfusion

Following initiation of perfusion at a flow rate of 25 $\mu\text{L}/\text{min}$, both cellular compartments remained morphologically stable throughout the experimental period. Daily microscopic monitoring was performed to assess cell attachment, morphology, and overall integrity of the co-culture model under continuous flow conditions.

Images acquired at the beginning of perfusion (Day 0) and after three days of continuous flow (Day 3) are shown in Figure 15. LSECs maintained a dense and adherent endothelial layer throughout the observation period. Similarly, differentiated HepaRG cells remained attached within the hepatic compartment and retained their characteristic morphology. No major cell detachment, disruption of the endothelial layer, or loss of HepaRG cultures was observed during daily brightfield microscopic examination throughout the perfusion period. These findings demonstrate that the established HepaRG–LSEC co-culture model tolerated the applied flow conditions and remained suitable for repeated medium exchange, daily sampling, and compound treatment experiments.

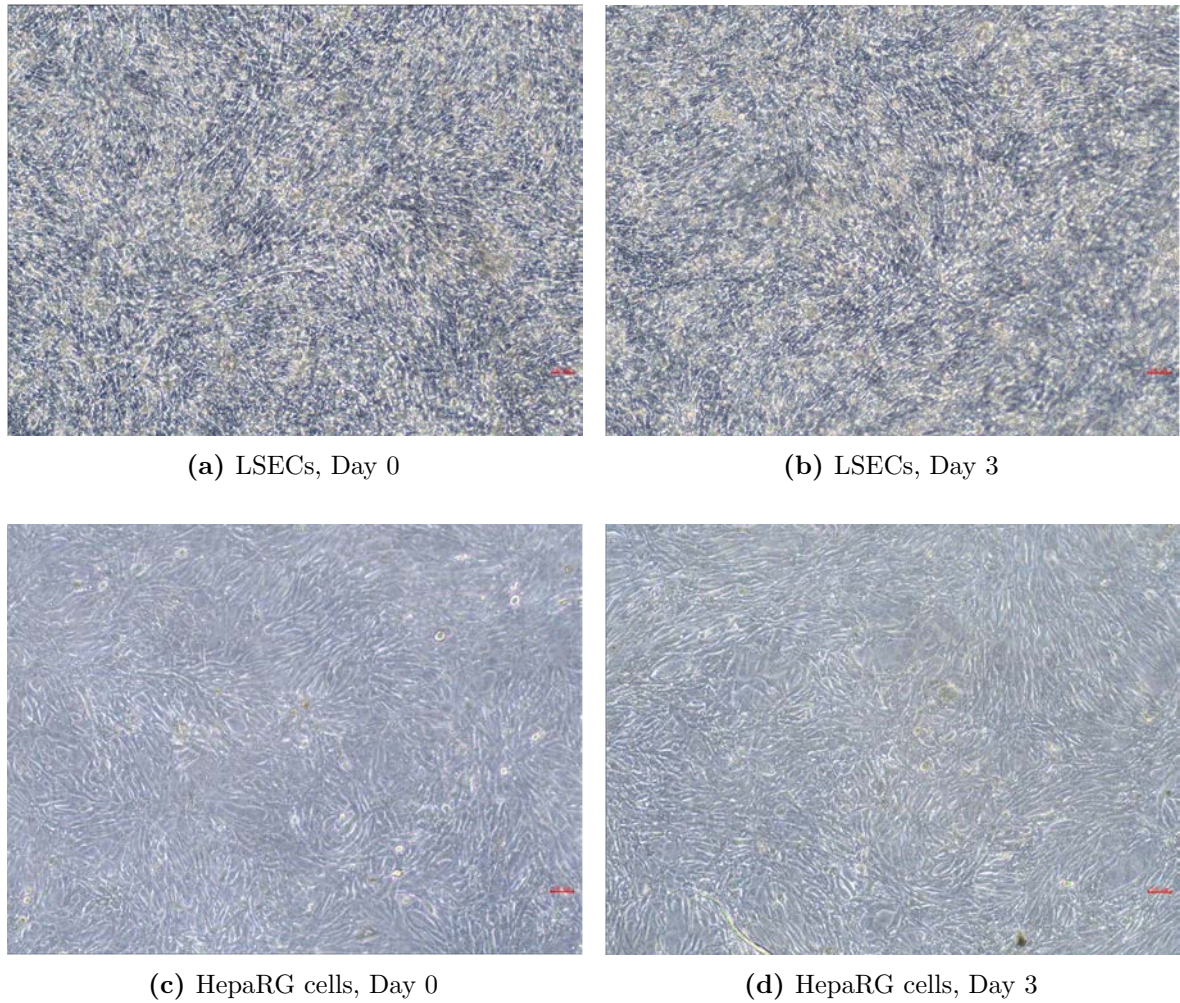


Figure 15: Maintenance of co-culture morphology during perfusion. Brightfield images of the final HepaRG–LSEC Liver-on-a-Chip model acquired immediately before initiation of perfusion (Day 0) and after three days of continuous flow (Day 3). Both LSECs and differentiated HepaRG cells remained adherent and maintained their characteristic morphology throughout the experimental period, indicating stable co-culture conditions under perfusion. Scale bars = 50 μm .

3.5 Morphological Assessment of Drug Treatments

Following confirmation of stable co-culture maintenance under control conditions (Section 3.4.2), treatment-related morphological changes were assessed by daily brightfield microscopy. Morphological changes were monitored throughout the experimental period to evaluate treatment-related alterations in cell attachment, monolayer integrity, and overall culture viability.

3.5.1 Staurosporine-Treated Cultures

Staurosporine was included as a positive control to induce cytotoxicity and to evaluate the ability of the Liver-on-a-Chip model to detect treatment-related cellular damage.

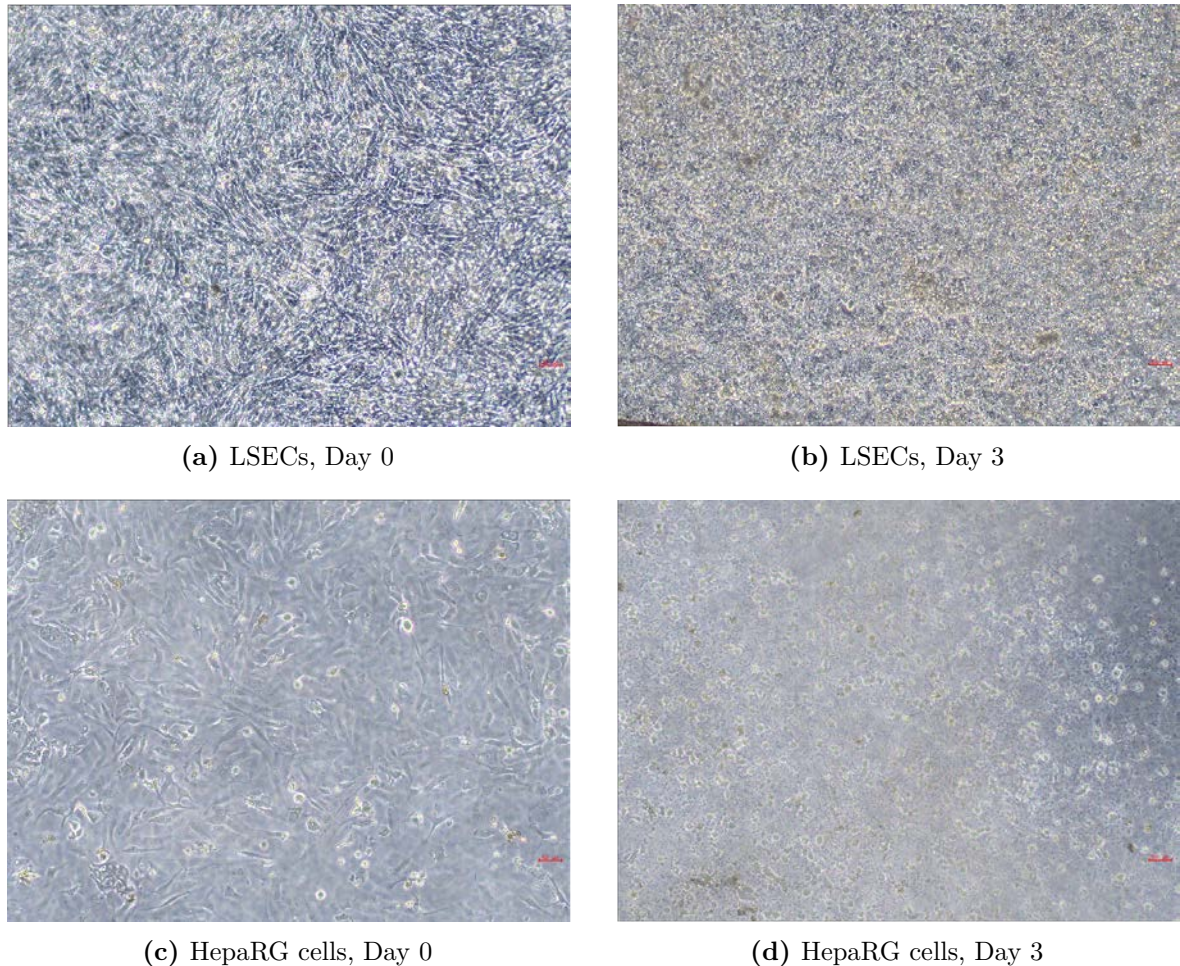


Figure 16: Morphological effects of staurosporine treatment in the HepaRG–LSEC Liver-on-a-Chip model. Brightfield images obtained immediately before treatment initiation (Day 0) and after three days of staurosporine exposure (Day 3). Both LSECs and HepaRG cells exhibited pronounced morphological alterations following treatment, including loss of cell density, disruption of monolayer integrity, and extensive cell detachment. Scale bars = 50 μm .

Morphological examination after three days of staurosporine exposure revealed substantial changes in both cellular compartments (Figure 16).

Compared with Day 0, LSECs displayed a marked reduction in cell density and loss of the characteristic confluent endothelial morphology. Similarly, HepaRG cultures exhibited extensive cell loss and disruption of the previously organized cellular structure. Numerous rounded and detached cells were observed in both compartments.

These morphological alterations were consistent with a pronounced cytotoxic response to staurosporine treatment.

3.5.2 Levofloxacin-Treated Cultures

To assess potential treatment-related morphological alterations, the HepaRG–LSEC Liver-on-a-Chip model was exposed to levofloxacin and monitored by brightfield microscopy over a period of three days.

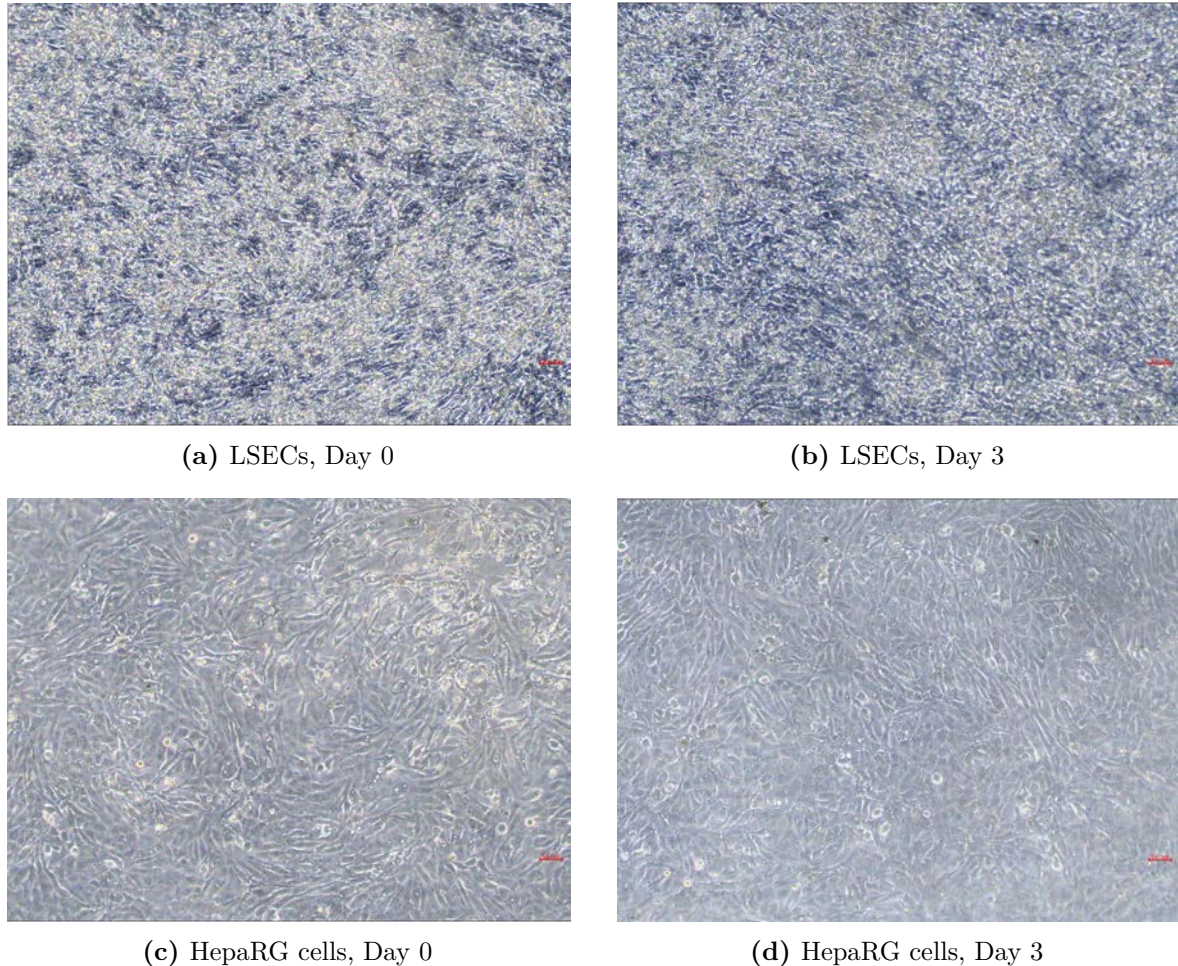


Figure 17: Morphological assessment of levofloxacin-treated HepaRG–LSEC cultures. Representative brightfield images obtained immediately before treatment initiation (Day 0) and after three days of levofloxacin exposure (Day 3). Both cellular compartments largely maintained their characteristic morphology throughout the treatment period. No pronounced loss of cell density, disruption of monolayer integrity, or extensive cell detachment was observed. Scale bars = 50 μm .

Morphological examination following three days of levofloxacin exposure revealed only minor changes in the cellular architecture of the Liver-on-a-Chip model (Figure 17). LSECs retained a largely confluent endothelial monolayer and preserved their characteristic elongated morphology throughout the treatment period. Similarly, HepaRG cultures remained densely populated and maintained their typical cellular organization. While minor local variations in confluency were observed, no substantial cell loss, widespread cell rounding, or extensive detachment was evident.

Overall, levofloxacin treatment did not induce pronounced morphological signs of cytotoxicity under the conditions tested.

3.5.3 Meropenem-Treated Cultures

To evaluate the potential effects of meropenem on cellular morphology, HepaRG–LSEC Liver-on-a-Chip cultures were monitored by brightfield microscopy before treatment initiation and after three days of exposure.

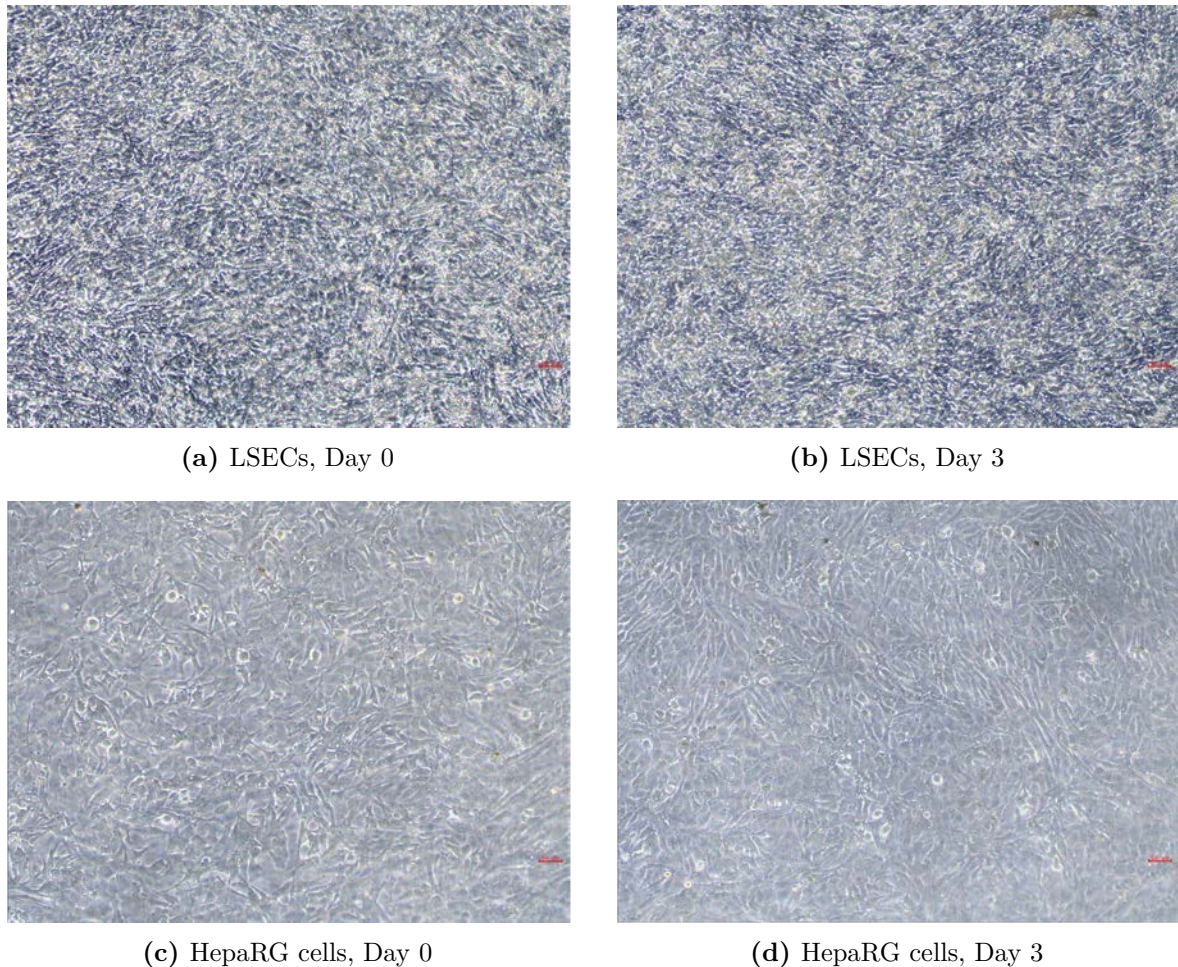


Figure 18: Morphological assessment of meropenem-treated HepaRG–LSEC cultures. Brightfield images obtained immediately before treatment initiation (Day 0) and after three days of meropenem exposure (Day 3). LSECs remained attached and formed a largely confluent cell layer throughout the treatment period, whereas HepaRG cells remained densely distributed with preserved cellular organization. No obvious cell rounding, marked reduction in cell density, disruption of the endothelial layer, or extensive cell detachment was observed. Scale bars = 50 μm .

Morphological examination after three days of meropenem exposure revealed no pronounced alterations in either cellular compartment of the Liver-on-a-Chip model (Figure 18).

LSECs maintained a dense and largely confluent endothelial layer with preservation of their characteristic elongated morphology. Likewise, HepaRG cultures remained well organized and exhibited no obvious signs of treatment-related deterioration. Cell density appeared comparable to that observed prior to treatment, and only minor variations in cellular appearance were noted.

Overall, meropenem treatment did not result in evident morphological signs of cytotoxicity under the experimental conditions applied.

3.6 LDH-Based Cytotoxicity Assessment

LDH release into the collected supernatants was quantified to assess treatment-induced cytotoxicity in both cellular compartments of the Liver-on-a-Chip model. Supernatants were collected daily throughout the treatment period, stored at -80°C , and analyzed after completion of the experiment. LDH values were normalized to the corresponding lysis controls.

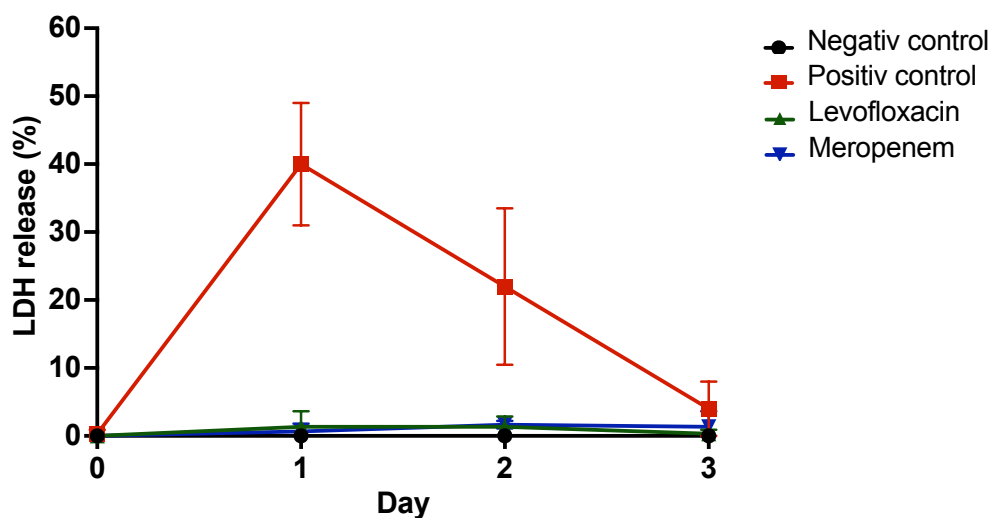


Figure 19: LDH release in the HepaRG compartment during compound treatment. LDH release was determined in daily supernatant samples collected from the HepaRG compartment over the three-day treatment period. Samples were stored at -80°C and analyzed after completion of the experiment. Staurosporine treatment resulted in a pronounced increase in LDH release compared with all other groups, whereas levofloxacin and meropenem showed LDH levels comparable to the negative control. Values are expressed as percentage of the corresponding lysis control and are presented as mean $\pm$ standard deviation (SD) from three independent experiments ($n = 3$). Error bars indicate the SD.

Figure 19 shows LDH release measured in the HepaRG compartment during the three-day treatment period. The positive control (staurosporine, red line) resulted in a pronounced increase in LDH release compared with all other groups. The highest LDH release was observed on Day 1, followed by a progressive decline on Days 2 and 3. In

contrast, the negative control (black line), levofloxacin (green line), and meropenem (blue line) remained at consistently low LDH levels throughout the experiment, indicating the absence of substantial treatment-induced cytotoxicity.

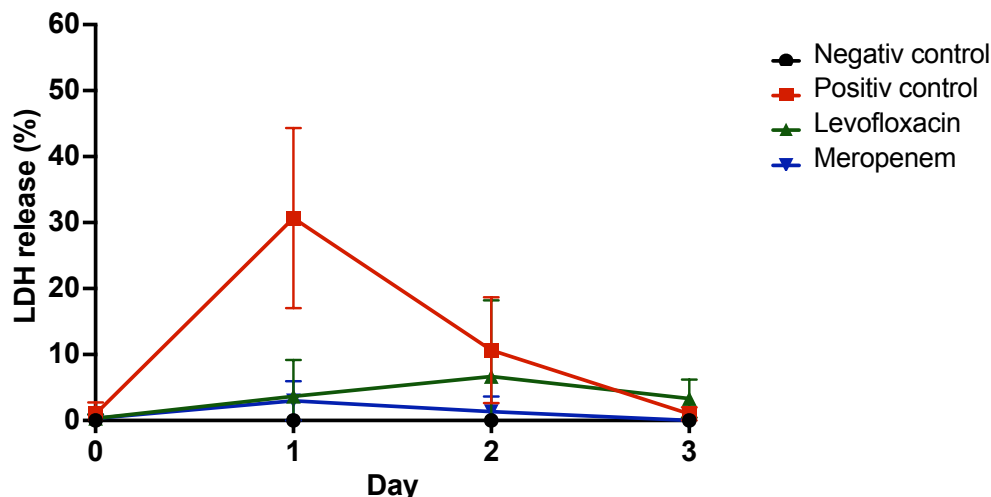


Figure 20: LDH release in the LSEC compartment during compound treatment. LDH release was determined in daily supernatant samples collected from the LSEC compartment over the three-day treatment period. Samples were stored at -80°C and analyzed after completion of the experiment. Increased LDH release was observed following staurosporine treatment, whereas levofloxacin and meropenem remained comparable to the negative control throughout the experiment. Values are expressed as percentage of the corresponding lysis control and are presented as mean $\pm$ standard deviation (SD) from three independent experiments ($n = 3$). Error bars indicate the SD.

LDH release measured in the LSEC compartment showed a similar temporal pattern (Figure 20). The positive control (staurosporine, red line) exhibited the highest LDH release on Day 1, followed by a gradual decline during the subsequent treatment period, confirming treatment-induced cellular damage within the endothelial compartment. In contrast, the negative control (black line), levofloxacin (green line), and meropenem (blue line) remained at low LDH levels throughout the experiment.

Overall, LDH measurements demonstrated a clear cytotoxic response to staurosporine treatment in both cellular compartments, whereas levofloxacin and meropenem produced no substantial increase in LDH release under the tested conditions. In both the HepaRG and LSEC compartments, LDH release peaked on Day 1 following staurosporine treatment and gradually declined over the remainder of the experimental period.

4 Discussion

4.1 Establishment of the Liver-on-a-Chip model

The primary objective of this study was to establish a stable and reproducible perfused Liver-on-a-Chip model that provides a human-relevant platform for future investigations of drug-induced liver injury (DILI). Rather than evaluating toxicological endpoints alone, this work focused on developing a technically robust microphysiological system that enables the controlled co-culture of hepatic and endothelial cells under physiologically relevant flow conditions.

The results demonstrate that differentiated HepaRG cells and immortalized human liver sinusoidal endothelial cells (LSECs) can be successfully cultured on opposite sides of a porous membrane under continuous microfluidic perfusion. Throughout the experimental period, both cell populations remained adherent, preserved their compartment-specific morphology, and tolerated continuous flow without evidence of impaired culture integrity. These findings indicate that the selected cell seeding strategy, extracellular matrix coating, culture conditions, and perfusion protocol together provide a reliable framework for maintaining stable hepatic and endothelial compartments within the Dynamic42 platform.

Reproducing two spatially separated yet functionally interconnected cellular compartments represents one of the key advantages of Liver-on-a-Chip systems over conventional static culture models. In the native liver, hepatocytes are separated from the sinusoidal blood flow by the fenestrated LSEC layer and the Space of Disse, allowing the continuous exchange of nutrients, metabolites, and signaling molecules while maintaining hepatocyte polarity and function (Moradi et al., 2020). Mimicking this microarchitecture under dynamic perfusion therefore provides a more physiologically relevant representation of the hepatic sinusoid than traditional two-dimensional cultures. In contrast, static culture systems lack vascular flow and endothelial interactions, conditions that promote the progressive loss of hepatocyte polarity, metabolic activity, and liver-specific functionality over time (Bell et al., 2018). Consequently, microphysiological liver models have emerged as promising tools for investigating liver physiology, xenobiotic metabolism, and hepatotoxicity with improved physiological relevance compared with conventional in vitro models (Bhatia & Ingber, 2014; Dalsbecker et al., 2022; Qiu et al., 2023).

The present work builds upon the Dynamic42 Liver-on-a-Chip platform established by Kaden et al. (Kaden, Graf, et al., 2023). The aim was to establish this previously described microphysiological platform using differentiated HepaRG cells and immortalized LSECs as a robust experimental basis for future DILI studies.

An important strength of the established model is the choice of differentiated HepaRG cells. Unlike many hepatic cell lines, differentiated HepaRG cells retain key characteristics of primary human hepatocytes, including stable expression of major drug-metabolizing enzymes such as CYP3A4, the formation of bile canaliculi, and long-term functional stability. These features make HepaRG cells particularly suitable for studies investigating xenobiotic metabolism and hepatotoxicity in microphysiological liver models (Guillouzo et al., 2007; Hart et al., 2010; Mayati et al., 2018).

Likewise, the use of liver sinusoidal endothelial cells increases the physiological relevance of the model compared with generic endothelial cell types such as human umbilical vein endothelial cells (HUVECs). LSECs are highly specialized endothelial cells that regulate substance exchange, immune homeostasis, and hepatocyte function within the hepatic sinusoid (DeLeve, 2015; Poisson et al., 2017). The immortalized LSECs employed in this study retain characteristic endothelial markers, including CD31, von Willebrand factor (vWF), and VE-cadherin, while offering the practical advantages of prolonged proliferative capacity and improved experimental reproducibility compared with primary LSECs.

Overall, the present study demonstrates the successful establishment of a stable and reproducible HepaRG–LSEC Liver-on-a-Chip model under continuous perfusion while preserving the structural integrity of both cellular compartments. Beyond maintaining tissue integrity, continuous microfluidic perfusion enables constant and clinically relevant drug exposure throughout the experimental period, thereby providing a more physiologically relevant representation of *in vivo* exposure conditions than conventional static cell culture. This aspect is particularly relevant for β -lactam antibiotics such as meropenem, whose efficacy depends on maintaining drug concentrations above the minimum inhibitory concentration ($fT > MIC$). Consequently, the established platform provides a solid foundation for future mechanistic investigations of drug-induced liver injury and represents an important step towards more physiologically relevant human *in vitro* liver models.

4.2 Selection of Model Compounds

To evaluate the biological functionality of the established Liver-on-a-Chip model, three compounds with distinct and well-characterized toxicity profiles were selected. Stauroporine served as a positive control to induce acute cytotoxicity, whereas levofloxacin and meropenem were chosen as clinically relevant antibiotics with low intrinsic hepatotoxic potential at therapeutic concentrations. This compound selection enabled assessment of whether the model reliably distinguishes between compounds expected to elicit pronounced cellular injury and those anticipated to preserve tissue integrity.

Staurosporine is a broad-spectrum protein kinase inhibitor that potently induces apoptosis through activation of intrinsic cell death pathways and is therefore widely employed as a positive control in studies investigating cytotoxicity and drug-induced liver injury (Belmokhtar et al., 2001; Kaden, Graf, et al., 2023). Consequently, pronounced morphological alterations accompanied by increased lactate dehydrogenase (LDH) release were expected in both the hepatic and endothelial compartments.

In contrast, levofloxacin and meropenem were deliberately selected because both antibiotics are clinically relevant anti-infective agents with a favorable hepatic safety profile at therapeutic concentrations. Levofloxacin, a widely prescribed fluoroquinolone with concentration-dependent antibacterial activity, and meropenem, a broad-spectrum β -lactam antibiotic whose efficacy depends on the duration for which free drug concentrations remain above the minimum inhibitory concentration ($fT > MIC$), represent two clinically important antibiotic classes with distinct pharmacokinetic and pharmacodynamic characteristics. Although rare cases of drug-induced liver injury have been reported for both compounds, these events are considered predominantly idiosyncratic rather than intrinsic and therefore occur only in susceptible individuals through complex interactions involving drug metabolism, immune activation, and genetic predisposition (Andrade et al., 2019). Such multifactorial mechanisms are difficult to recapitulate in simplified *in vitro* systems that lack the full complexity of the hepatic microenvironment (Andrade et al., 2019; Hoofnagle & Björnsson, 2019). Meropenem was of particular interest because β -lactam antibiotics are frequently administered as prolonged or continuous infusions in clinical practice, making them particularly suitable compounds for evaluating drug responses under continuous perfusion.

To approximate clinically relevant exposure conditions, both antibiotics were administered at concentrations corresponding to reported plasma C_{max} values rather than at supraphysiological doses. This experimental strategy enabled assessment of the biological response of the established Liver-on-a-Chip model under clinically relevant exposure conditions.

4.3 Evaluation of Drug-Induced Cytotoxicity in the Established Liver-on-a-Chip Model

To determine whether the established HepaRG–LSEC Liver-on-a-Chip model responded to the selected compounds as anticipated, morphological assessment was combined with quantification of lactate dehydrogenase (LDH) release. Together, these complementary readouts enabled evaluation of the model’s ability to discriminate between compounds with distinct and well-defined toxicity profiles.

The combination of qualitative morphological assessment and quantitative LDH measurements represents a complementary strategy for evaluating acute cytotoxicity. While brightfield microscopy enables visualization of structural alterations and compartment-specific cellular responses, LDH release provides an objective biochemical measure of plasma membrane damage and irreversible cell death (Chan et al., 2013).

As expected, staurosporine induced pronounced cytotoxicity in both cellular compartments. Brightfield microscopy revealed extensive cell rounding, loss of cell adhesion, disruption of the endothelial monolayer, and marked deterioration of the HepaRG cultures following three days of treatment. These morphological alterations were paralleled by a substantial increase in LDH release, indicating loss of plasma membrane integrity and irreversible cell damage. The close agreement between microscopic observations and biochemical measurements demonstrates that the established model reliably detects acute treatment-induced cellular injury under continuous perfusion and confirms LDH release as a robust indicator of cytotoxicity within the microfluidic system.

When interpreting the temporal LDH profiles, the release kinetics of the enzyme should be considered. Because LDH is passively released only after disruption of the plasma membrane, it reflects irreversible cell death rather than reversible cellular stress (Chan et al., 2013). Consequently, each cell contributes to the extracellular LDH pool only once. Following extensive cell death during the early phase of treatment, progressively fewer viable cells remain capable of releasing additional LDH, resulting in declining enzyme levels despite the persistence of severe morphological damage. The decrease in LDH observed at later time points should therefore be interpreted as a consequence of cellular depletion rather than recovery of the culture.

Although LDH release provided a sensitive and quantitative measure of membrane damage, it did not capture the full spectrum of treatment-induced cellular alterations. LDH measurements alone cannot distinguish between different types of structural damage, identify changes in cell morphology or monolayer integrity, or reveal compartment-specific alterations within the microfluidic co-culture. Brightfield microscopy therefore provided essential complementary information by allowing direct visualization of cell morphology, cellular organization, and the extent of cell detachment. The close agreement between morphological observations and LDH release in the present study substantially strengthened the interpretation of the experimental findings and provided a more comprehensive assessment of compound-induced cytotoxicity than either method alone. Interestingly, LDH release was consistently more pronounced in the HepaRG compartment than in the endothelial compartment. This compartment-specific response is consistent with the central role of hepatocytes as the primary target of many hepatotoxic compounds.

In contrast, treatment with levofloxacin and meropenem resulted in neither substantial

morphological alterations nor increased LDH release compared with untreated controls. Throughout the experimental period, both the endothelial and hepatic compartments largely preserved their structural integrity, indicating that the established co-culture remained stable under clinically relevant antibiotic exposure. These findings are consistent with the low intrinsic hepatotoxic potential of both compounds at therapeutic concentrations. Although rare cases of liver injury have been reported clinically, these adverse reactions are predominantly idiosyncratic and involve complex interactions between drug metabolism, immune activation, and individual susceptibility rather than direct dose-dependent cytotoxicity (Andrade et al., 2019). Accordingly, the absence of measurable toxicity under the present experimental conditions supports the specificity of the model and suggests that it does not generate false-positive cytotoxic responses when exposed to compounds with low intrinsic hepatotoxic potential.

Overall, the observed response pattern demonstrates that the established Liver-on-a-Chip model reliably discriminates between compounds with expected cytotoxic and non-cytotoxic profiles. The pronounced response to staurosporine, together with the absence of detectable toxicity following treatment with levofloxacin and meropenem, confirms the biological functionality of the model and provides confidence in its application for future studies investigating mechanisms of drug-induced liver injury. The relationship of these findings to previously published Liver-on-a-Chip models is discussed in the following section.

4.4 Comparison with Previously Published Liver-on-a-Chip Models

The findings of the present study are consistent with a growing body of literature demonstrating that microphysiological Liver-on-a-Chip systems provide a more physiologically relevant *in vitro* environment than conventional static cultures for studying liver function, drug metabolism, and hepatotoxicity. By integrating controlled microfluidic perfusion with multicellular co-culture, these platforms more closely recapitulate the structural and functional characteristics of the hepatic sinusoid, thereby improving the maintenance of hepatic phenotype and enabling more predictive assessment of drug responses (Fu et al., 2023; Otumala et al., 2023; Qiu et al., 2023). Consequently, Liver-on-a-Chip technology has emerged as a promising strategy to bridge the translational gap between conventional cell culture models and human physiology.

The present work builds upon the Dynamic42 Liver-on-a-Chip platform originally established by Kaden et al. In their initial study, a perfused dual-channel liver model comprising expandable upcyte[®] hepatocytes and liver sinusoidal endothelial cells (LSECs)

was successfully employed to investigate acetaminophen-induced liver injury (Kaden, Noerenberg, et al., 2023). Subsequently, the platform was further developed by replacing the hepatocytes with differentiated HepaRG cells and incorporating monocyte-derived macrophages (MDMs), thereby increasing the physiological complexity of the model and enabling investigation of antibiotic-induced liver injury caused by trovafloxacin and levofloxacin (Kaden, Graf, et al., 2023). In contrast, the present study focused on establishing a simplified HepaRG–LSEC co-culture as a robust and reproducible foundation for future DILI studies.

Despite these differences in biological complexity, several key observations were highly consistent across the published Dynamic42 models. Similar to the previous studies, stable compartmentalized co-cultures were successfully maintained under continuous perfusion, while both the hepatic and endothelial compartments preserved their morphology and structural integrity throughout the experimental period. These findings further support the robustness and reproducibility of the Dynamic42 platform and demonstrate that it can be successfully implemented using differentiated HepaRG cells and immortalized LSECs.

The observed cytotoxicity pattern also closely resembled previous reports. In the acetaminophen study by Kaden et al., toxic injury was associated with substantially higher LDH release in the hepatic compartment than in the endothelial compartment, reflecting the greater susceptibility of hepatocytes to chemically induced injury (Kaden, Noerenberg, et al., 2023). A comparable compartment-specific response was observed following staurosporine treatment in the present study. Although direct quantitative comparison is not possible due to the different toxicants employed, the similar distribution of cellular injury between the two compartments suggests that the established model reproduces biologically meaningful injury patterns consistent with previously validated Dynamic42 Liver-on-a-Chip models.

The response to levofloxacin likewise agrees well with previous observations. Kaden et al. demonstrated that clinically relevant concentrations of levofloxacin induced little or no measurable cytotoxicity, whereas the structurally related antibiotic trovafloxacin produced pronounced liver injury in their immunocompetent HepaRG–LSEC–MDM model (Kaden, Graf, et al., 2023). The absence of detectable toxicity observed in the present study at clinically relevant concentrations therefore further supports the biological validity of the established HepaRG–LSEC co-culture and indicates that the model appropriately distinguishes compounds with low intrinsic hepatotoxic potential from those inducing acute cellular injury.

Importantly, these observations are not limited to the Dynamic42 platform. Numerous Liver-on-a-Chip studies have demonstrated that the combination of physiological perfusion and multicellular liver-specific co-cultures can improve the maintenance of

hepatic phenotype, metabolic competence, and long-term tissue stability compared with conventional static culture systems (Fu et al., 2023; Otumala et al., 2023; Qiu et al., 2023). Moreover, several studies have shown that microphysiological liver models are capable of discriminating between hepatotoxic and non-hepatotoxic compounds with greater physiological relevance than traditional two-dimensional cultures, highlighting their increasing value for preclinical drug safety assessment and mechanistic DILI research (Fu et al., 2023; Qiu et al., 2023).

Nevertheless, an important distinction between the present model and more advanced Liver-on-a-Chip systems should be acknowledged. While the HepaRG–LSEC co-culture successfully reproduces essential structural features of the hepatic sinusoid, it lacks resident immune cells that contribute substantially to inflammatory signalling and the pathogenesis of idiosyncratic drug-induced liver injury. As demonstrated by Kaden et al., the incorporation of monocyte-derived macrophages enables investigation of immune-mediated mechanisms that cannot be captured in the present two-cell-type model (Kaden, Graf, et al., 2023). Similar efforts by other groups increasingly emphasize the importance of integrating Kupffer cells, stellate cells, and additional non-parenchymal liver cell populations to further improve physiological relevance and enable modelling of complex liver diseases and immune-mediated DILI (Otumala et al., 2023; Qiu et al., 2023).

Overall, the close agreement between the present findings, previous Dynamic42 studies, and the broader Liver-on-a-Chip literature demonstrates that the established HepaRG–LSEC model reproduces the characteristic behaviour reported for modern microphysiological liver systems. At the same time, its comparatively simple yet robust design provides a reproducible experimental platform that can readily serve as the basis for future incorporation of additional liver-specific cell types and more sophisticated investigations of drug-induced liver injury.

4.5 Limitations of the Model and Future Perspectives

Although the present study successfully established a stable and reproducible perfused HepaRG–LSEC Liver-on-a-Chip model, several aspects require further investigation before the platform can be considered a comprehensive model for studying drug-induced liver injury (DILI). Addressing these limitations will further strengthen the biological relevance and predictive potential of the system.

One important consideration concerns the transfer of differentiated HepaRG cells from conventional culture into the microfluidic chip. To enable seeding of the hepatic com-

partment, differentiated HepaRG cells were enzymatically detached by trypsinization, a process that inevitably disrupts cell–cell junctions and may transiently affect cellular polarity, differentiation status, and liver-specific functionality. Although the characteristic morphology of differentiated HepaRG cultures was preserved following transfer, morphology alone cannot confirm maintenance of the differentiated hepatic phenotype or metabolic competence. Since the predictive value of HepaRG cells largely depends on their preserved expression of drug-metabolizing enzymes and liver-specific functions, additional validation of their functional phenotype after chip seeding is warranted (Aninat et al., 2006; Guillouzo et al., 2007; Mayati et al., 2018).

Future studies should therefore complement morphological assessment with functional characterization of the hepatic compartment. Quantification of liver-specific markers, including CYP3A4, CYP2E1, albumin, HNF4 α , and ASGPR1, by RT-qPCR or immunofluorescence would provide valuable information regarding maintenance of the differentiated phenotype. In addition, direct assessment of cytochrome P450 enzyme activity would verify whether the cells retain their metabolic capacity following enzymatic detachment and transfer into the microfluidic environment. Such analyses would substantially strengthen confidence in the biological functionality of the established model and its suitability for drug metabolism and hepatotoxicity studies (Guillouzo et al., 2007; Mayati et al., 2018)..

A practical strategy to address this question would be to perform complementary experiments in conventional multiwell plates before transferring the cells into the chip. Direct comparison of differentiated HepaRG cells before and after trypsinization with respect to morphology, gene expression, and CYP activity would represent a straightforward and cost-effective approach to optimize the transfer procedure prior to conducting more resource-intensive microfluidic experiments.

A second limitation concerns the functional characterization of the established model. In the present study, biological validation relied primarily on brightfield microscopy and lactate dehydrogenase (LDH) release, both of which represent robust indicators of acute cytotoxicity. However, these endpoints provide only limited insight into hepatocyte-specific function and the mechanisms underlying cellular injury. Future studies should therefore incorporate additional functional readouts, including albumin secretion, urea synthesis, ATP content, reactive oxygen species production, mitochondrial function, bile canalicular integrity, and inflammatory cytokine release. Combining multiple complementary endpoints would enable a more comprehensive assessment of hepatic function, improve the predictive value of the platform for preclinical hepatotoxicity testing, and facilitate mechanistic investigations of DILI (Fu et al., 2023; Otumala et al., 2023; Qiu et al., 2023).

An additional aspect that should be considered is the actual drug exposure within

the microfluidic system. Although the antibiotics were administered at concentrations corresponding to reported plasma $C_{\max}$ values, their concentrations within the chip and the perfusate were not analytically determined during the experiments. Adsorption of compounds to polymeric materials, degradation, or other system-related losses may alter the effective concentration reaching the cells over time. Future studies should therefore include analytical quantification of drug concentrations, for example by LC–MS/MS, to verify the actual cellular exposure and further validate the predictive performance of the established Liver-on-a-Chip model.

Finally, the limited number of independent biological experiments should also be acknowledged as a limitation. As the primary objective of this work was to establish and technically validate the Liver-on-a-Chip platform, biological validation was based on three independent experiments. Due to this limited sample size, no inferential statistical analyses were performed, and the findings were therefore evaluated descriptively in conjunction with the morphological assessment. While this approach was sufficient to demonstrate the feasibility, reproducibility, and overall functionality of the model, it limits the statistical robustness of the findings and the extent to which broader biological conclusions can be drawn. Future studies should therefore include a larger number of independent biological experiments to enable meaningful statistical analyses and further strengthen confidence in the predictive performance of the established platform.

Finally, while the established HepaRG–LSEC co-culture reproduces essential structural features of the hepatic sinusoid, it still represents a simplified version of the native liver microenvironment. DILI, particularly its idiosyncratic forms, is increasingly recognized as a multicellular process involving complex interactions between hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, cholangiocytes, and infiltrating immune cells (Andrade et al., 2019; Poisson et al., 2017). Consequently, incorporating additional liver-specific cell populations represents an important next step in the further development of the platform. As demonstrated by Kaden et al., integration of monocyte-derived macrophages enables investigation of inflammatory mechanisms that cannot yet be reproduced in the present two-cell-type model (Kaden, Graf, et al., 2023). Likewise, recent Liver-on-a-Chip studies increasingly emphasize that inclusion of multiple non-parenchymal cell types substantially improves the physiological relevance of microphysiological liver systems and expands their applicability for modelling complex liver diseases and immune-mediated DILI (Otumala et al., 2023; Qiu et al., 2023).

Taken together, the present work establishes a technically robust and reproducible HepaRG–LSEC Liver-on-a-Chip model that provides a solid foundation for future investigations of hepatic drug responses under dynamic perfusion conditions. While additional functional validation and increased biological complexity will be required, the successful implementation of this microphysiological platform provides a robust

foundation for the continued development of more predictive, human-relevant in vitro models for studying drug-induced liver injury. Continued refinement of the model, particularly through the integration of additional liver-specific cell types and comprehensive functional characterization, has the potential to improve preclinical safety assessment, facilitate mechanistic studies of DILI, and ultimately contribute to reducing reliance on conventional animal models.

5 Conclusion

In conclusion, the present study successfully established a stable and reproducible human Liver-on-a-Chip model comprising differentiated HepaRG cells and liver sinusoidal endothelial cells cultured under continuous microfluidic perfusion. Both cellular compartments remained viable and structurally intact throughout the experimental period, demonstrating that the selected culture conditions, cell seeding strategy, and perfusion protocol provide a robust basis for reproducible microphysiological co-culture experiments.

Biological validation confirmed that the established model reliably discriminates between compounds with expected cytotoxic and non-cytotoxic profiles. As anticipated, staurosporine induced pronounced morphological deterioration accompanied by increased LDH release, whereas levofloxacin and meropenem did not elicit detectable acute cytotoxicity under clinically relevant exposure conditions. Together, these findings demonstrate that the model responds appropriately to compounds with distinct toxicity profiles while maintaining stability under physiological perfusion conditions.

Although additional functional characterization of the hepatic phenotype and incorporation of further liver-specific cell populations will be necessary to further enhance the physiological complexity of the platform, the present work provides a robust foundation for future investigations of drug-induced liver injury. By establishing a technically reliable and biologically functional HepaRG–LSEC Liver-on-a-Chip model, this study contributes to the ongoing development of more predictive, human-relevant *in vitro* systems that may improve preclinical hepatotoxicity assessment, facilitate mechanistic DILI research, and ultimately support the reduction of animal experimentation in drug development.

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