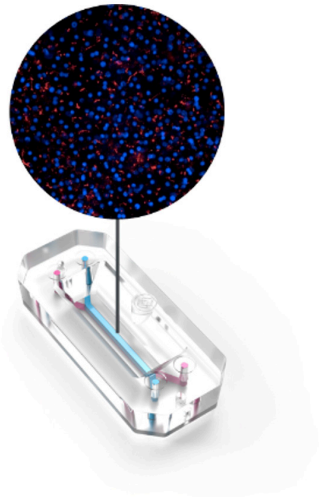
Emulate Liver-Chip R1: A human-relevant model of the liver sinusoid using a novel, low-drug-absorbing chip



Key Highlights

- The Chip-R1™ Rigid Chip has the same two-channel configuration as the Chip-S1® Stretchable Chip, with several updates, including reduced drug absorption.
- The Liver-Chip R1 demonstrates robust liver functionality, as indicated by morphology, marker expression, albumin production, and drug metabolism.
- The Liver-Chip R1 displays increased sensitivity to detecting the drug-induced liver injury risk of small-molecule drugs with absorption liability in PDMS.
- The Chip-R1 exhibits reduced absorption of a range of small molecules with diverse physicochemical properties.

Introduction

Drug development is a lengthy and expensive process, often taking over 10 years and costing ~$2B to bring a new drug to market (Deloitte, 2023). Current preclinical safety testing relies on conventional *in vitro* models that often fail to adequately replicate the *in vivo* 3D architecture and physiological environment or animal models that do not accurately predict human responses. These limitations result in poor clinical translation, with around 90% of drugs failing in clinical trials (Mullard, 2016).

To create a more human-relevant research model, Emulate has previously developed the Chip-S1® Stretchable Chip and associated organ models, including the liver, kidney, duodenum, and colon. The Chip-S1 is made from polydimethylsiloxane (PDMS), a material chosen for its biocompatibility, gas permeability, optical clarity, and stretchability, enabling researchers to model the mechanical forces induced by breathing or peristalsis. However, PDMS has the potential for drug absorption, which can diminish the system's ability to accurately predict the toxicity, efficacy, and pharmacokinetics for a subset of hydrophobic small-molecule drug candidates.

To address these challenges, Emulate developed the Chip-R1 Rigid Chip, which minimizes drug absorption while maintaining the essential architecture of Chip-S1. This enables researchers to build biologically complex Organ-Chip models for tissues that do not require stretch (e.g., liver, kidney, brain, and lung airway). Here, we describe the development of the Chip-R1 Rigid Chip and the Liver-Chip R1 organ model, including data from an equivalency study that demonstrates its utility in modeling the liver and predicting drug hepatotoxicity.

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Goal

To develop a minimally drug-absorbing Organ-Chip and characterize its application for modeling the human liver sinusoid for preclinical drug hepatotoxicity testing.

Materials

Hardware

- Zoë-CM1® or Zoë-CM2® Culture Module
- Orb® Hub Module

Consumables

- Liver-Chip R1 BioKit: Chip-R1 Rigid Chips, Pod-2™ Portable Module, and primary human cells (hepatocytes, stellate cells, Kupffer cells, and liver sinusoidal endothelial cells (LSECs))

Drugs for Evaluation:

- Hepatotoxicity:
  - Nefazodone hydrochloride
  - Levofloxacin
  - Trovafloxacin mesylate
- Cytochrome P450 activity:
  - Phenacetin
  - Diclofenac sodium
  - Bufuralol hydrochloride
  - Midazolam hydrochloride

Chip-R1 and Pod-2 Design

The Chip-R1 maintains the two-channel architecture of the Chip-S1 and has several enhancements to improve performance, including updated chip materials, decreased vascular channel height, and reduced chip membrane pore diameter. The system also includes an updated media cartridge, the Pod-2 Portable Module.

Key updates include:

- **Minimal drug absorption:** The Chip-R1 and associated Pod-2 are made of cyclic olefin polymer (COP), polycarbonate (PC), polyethylene terephthalate (PET), and other minimally absorbing materials to reduce the impact of drug absorption. The change to rigid thermoplastics makes the consumable suitable for non-stretch models.
- **Increased maximum shear stress:** A shorter bottom channel in Chip-R1 and updated microfluidic elements in Pod-2 combine to increase the maximum shear stress in the bottom channel (up to 2.3 dyn/cm2 at a flow rate of 2,000 µL/h).
- **Updated membrane:** The Chip-R1 features a smaller pore diameter (R1: 3 µm; S1: 7 µm) as well as a thinner membrane (R1: 22 µm; S1: 50 µm).
- **Simplified workflow:** The tissue-culture-treated membrane eliminates the need for cchip activation prior to extracellular matrix coating.

See **Figure 1** for representative schematics and images of the Chip-R1 and Pod-2 architecture. See **Table 1** for a full comparison of the Chip-S1 and Chip-R1 architecture.

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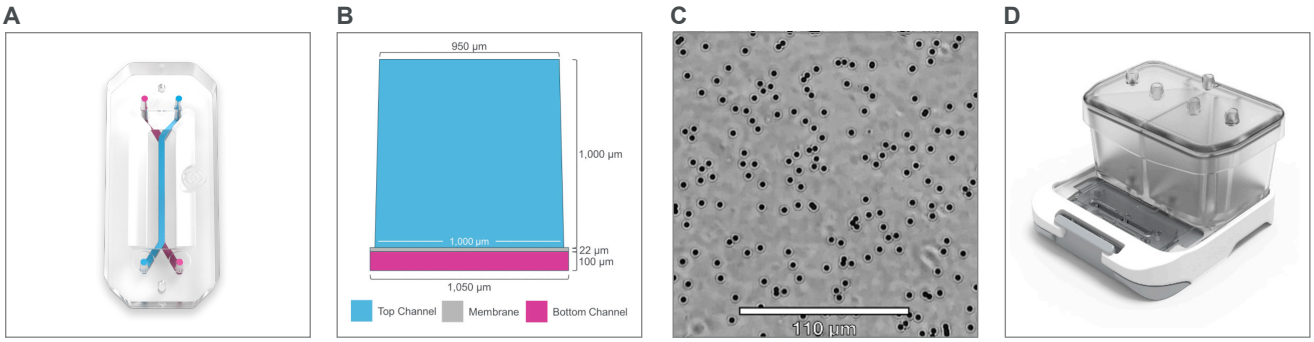

***Figure 1. A**) 3D model of Chip-R1. **B**) Schematic of Liver-Chip R1 with cell culture channel dimensions. **C**) Phase contrast image of PC membrane used for cell culture (scale bar: 110 µm) **D**) 3D model of Pod-2.*

	Chip-S1	Chip-R1
Bottom channel height	200 µm	100 µm
Maximum flow rate	1,000 µL/h	2,000 µL/h
Maximum bottom channel shear	0.3 dyn/cm^2	2.3 dyn/cm^2
Membrane thickness	50 µm, PDMS	22 µm, PC
Membrane pores	7 µm diameter, hexagonally packed	3 µm diameter, random distribution
Membrane porosity	2.7%	2.8%
Imaging distance *(bottom of chip to top of membrane)*	850 µm	172 µm
Chip Activation Required	Yes (ER-1® and ER-2® Activation Reagents)	No (Tissue-culture treated membrane)
Stretchable membrane	Yes	No
Associated Pod® Portable Module	Pod-1®	Pod-2 (decreased absorption)

***Table 1.** Chip-R1 vs. Chip-S1 Comparison.*

Results

Liver-Chip R1 Model Characterization

An equivalency study was performed to assess the biological performance of the Chip-R1 compared to the Chip-S1, using the Liver-Chip as the reference biological model.

To prepare the Liver-Chip R1, the top and bottom channels were coated with a tissue-specific extracellular matrix (ECM) before primary human hepatocytes were seeded in the top channel. On the same day as hepatocyte seeding, a Matrigel overlay was introduced in the top channel to promote a three-dimensional matrix for the hepatocytes to grow in an ECM sandwich culture.

After an incubation period of 1 day, the bottom channel was seeded with non-parenchymal cells (NPCs)—specifically primary human liver sinusoidal endothelial cells (LSECs), Kupffer cells, and stellate cells. This arrangement emulates the *in vivo* hepatocyte-sinusoidal endothelial interface. After incubating for 4 hours, chips were then placed in Zoë Culture Module under 30 µL/h media flow for the duration of the experiment.

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Results (continued)

After the 5-6 day stabilization period, the Liver-Chip models were assessed for liver-specific functionality over 7 days (monitoring phase). All readouts are referenced to timepoint days (TPD), with TPD0 being the beginning of this experimental phase. Analysis showed that both the Liver-Chip R1 and Liver-Chip S1 replicated key structural and functional aspects of the liver sinusoid for up to 7 days of monitoring, as assessed by morphology, immunofluorescent staining, albumin production, and liver metabolic activity.

Phase contrast microscopy of Liver-Chip R1 revealed a healthy monolayer of hepatocytes, indicated by a cuboidal and binucleated morphology, and non-parenchymal cells, evident by a confluent monolayer in the bottom channel (see **Figure 2**).

Confocal immunofluorescence microscopy confirmed liver-specific morphological structures, indicated by the presence of multidrug resistance-associated protein 2 (MRP2), Stabilin-2 expression in LSECs, and alpha-smooth muscle actin expression in stellate cells (see **Figure 3**).

The baseline fidelity and robustness of Liver-Chip R1 was assessed compared to Liver-Chip S1, utilizing albumin production as a key functional biomarker of hepatocellular health. The Liver-Chip R1 demonstrated physiologically relevant levels of albumin synthesis (threshold minimum of 20 µg per day per million hepatocytes) for up to 7 days during the experimental phase under control conditions (see **Figure 4**).

Functionality of the Liver-Chip R1 was further assessed by measuring the activity of key cytochrome P450 (CYP) metabolizing enzymes (see **Figure 5**). A cocktail preparation of four probe substrates (phenacetin, diclofenac, bufuralol, and midazolam) was administered to determine metabolic competency of the major CYP isoforms (CYP1A2, CYP2C9, CYP2D6, and CYP3A4, respectively) associated with drug metabolism. This drug cocktail was flowed on chip at 150 µL/h for 1 hour to minimize the potential for metabolite-induced feedback inhibition from the metabolizing enzymatic activity. LC/MS assessment of parent-to-metabolite production showed comparable levels of enzymatic activity between Liver-Chip R1 and Liver-Chip S1. These results suggest the Liver-Chip R1 has healthy rates of CYP metabolic activity, an essential hepatocyte function which plays an important role in liver health, disease, and drug toxicity.

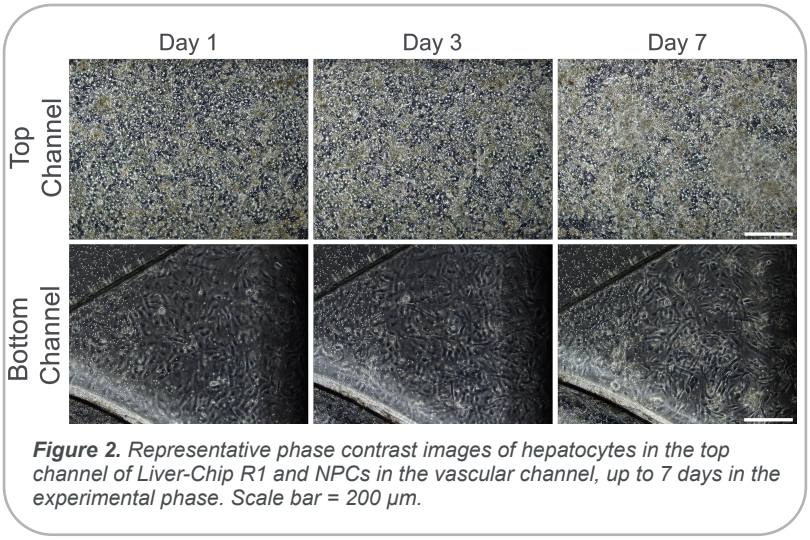

Figure 2. *Representative phase contrast images of hepatocytes in the top channel of Liver-Chip R1 and NPCs in the vascular channel, up to 7 days in the experimental phase. Scale bar = 200 µm.*

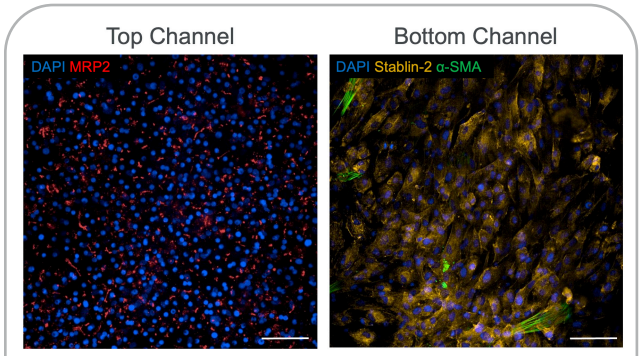

Figure 3. *Day 0 representative immunofluorescent images of the Liver-Chip R1 showing MRP2-containing bile canaliculi. Day 7 representative immunofluorescent images of the Liver-Chip R1 showing Stabilin-2-stained liver sinusoidal endothelial cells and alpha-smooth muscle actin-stained stellate cells. Scale bar = 100 µm.*

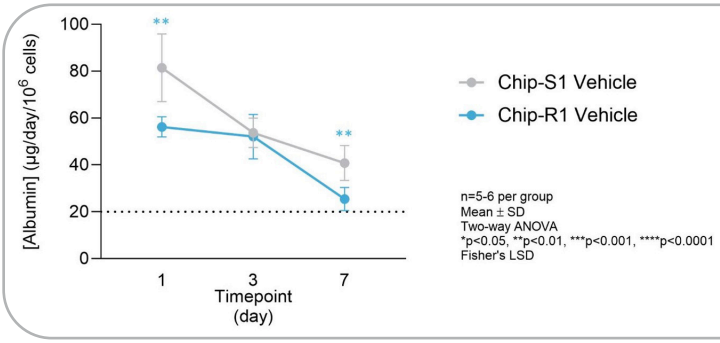

Figure 4. *Albumin secretion on days 1, 3, and 7 of the experimental phase under control conditions, measured by ELISA. Dotted line represents healthy albumin acceptance criteria of 20 µg/day/million cells. showing Stabilin-2-stained liver sinusoidal endothelial cells and alpha-smooth muscle actin-stained stellate cells. Scale bar = 100 µm.*

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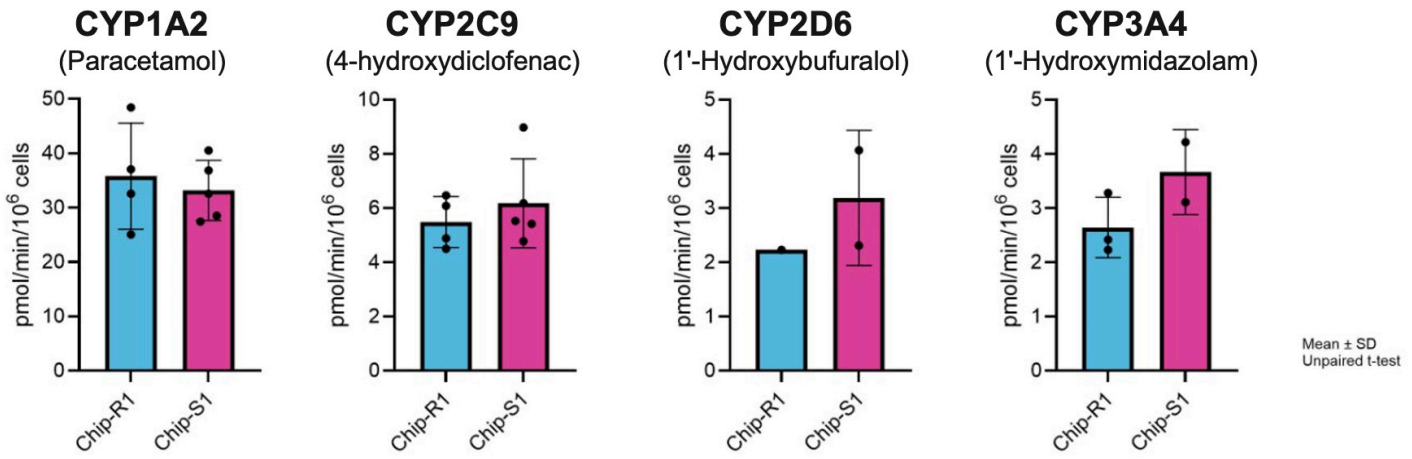

Figure 5. *CYP cocktail metabolite formation, assessed 1 hour post-dosing at 150 µL/h and measured via liquid chromatography-mass spectrometry. n = 4 chips/group for Chip-R1 and 5 chips/group for Chip-S1. Metabolite levels could not be determined for certain data points due to assay sensitivity and metabolite formation levels close to the lower limit of quantification.*

Liver-Chip R1 Toxicity Testing

One of the major advantages of Liver-Chip S1 is its predictive validity in detecting drug-induced liver injury (DILI) in hepatotoxic compounds (Ewart et al., 2022[2]). To assess whether the Liver-Chip R1 offers similar or enhanced sensitivity, a comparative study was conducted using three compounds with different toxicity and PDMS absorption profiles.

Nefazodone, a highly hepatotoxic compound with high PDMS absorption, was administered at 3, 10, and 30 µM. Additionally, the structural analogs trovafloxacin and levofloxacin were tested, both compounds with minimal PDMS absorption. Trovafloxacin is a known hepatotoxic compound with a high ranking on the FDA and Garside DILI severity scales, while levofloxacin is less toxic (Garside et al., 2014[3]). Trovafloxacin and levofloxacin were administered at 200 and 537 µM, respectively.

The Chip-R1 demonstrated greater sensitivity in detecting DILI risk of the highly absorbing drug nefazodone, demonstrating the model's reduced drug absorption properties. When treated with 30 µM nefazodone at the protocol-recommended flow rate of 30 µL/h, the Liver-Chip R1 demonstrated significant signs of hepatotoxicity, with observable cell death via phase contrast imaging and morphology scoring (see **Figure 6**), as well as a reduction in albumin production and an increase in alanine transaminase (ALT) release (see **Figures 7** and **8**) (Ewart et al., 2022[2]). In contrast, the Liver-Chip S1 treated with the same concentration and flow rate of nefazodone showed minimal signs of DILI, with albumin, ALT production, and morphology scoring comparable to the vehicle-treated Liver-Chip S1. The increased sensitivity of the Chip-R1 allows for greater accuracy in predicting an IC50 value for nefazodone compared to the Chip-S1 consumable due to high absorption of nefazodone into PDMS at all concentrations tested (see **Figure 7**). This highlights the utility of the Chip-R1 consumable for studies aiming to predict the toxicity of compounds which may be highly liable to PDMS absorption.

Additionally, the Liver-Chip R1 maintains comparable sensitivity to Liver-Chip S1 in discriminating more hepatotoxic compounds, from less hepatotoxic ones, as demonstrated by treatment with trovafloxacin and levofloxacin. Both Liver-Chip-R1 and Liver-Chip S1 demonstrated significant signs of DILI when dosed with trovafloxacin, including a reduction in albumin production and increases in ALT release. In contrast, levofloxacin resulted in fewer signs of DILI, with both chips demonstrating healthier levels of albumin and ALT production compared to trovafloxacin-treated chips (see **Figure 9**).

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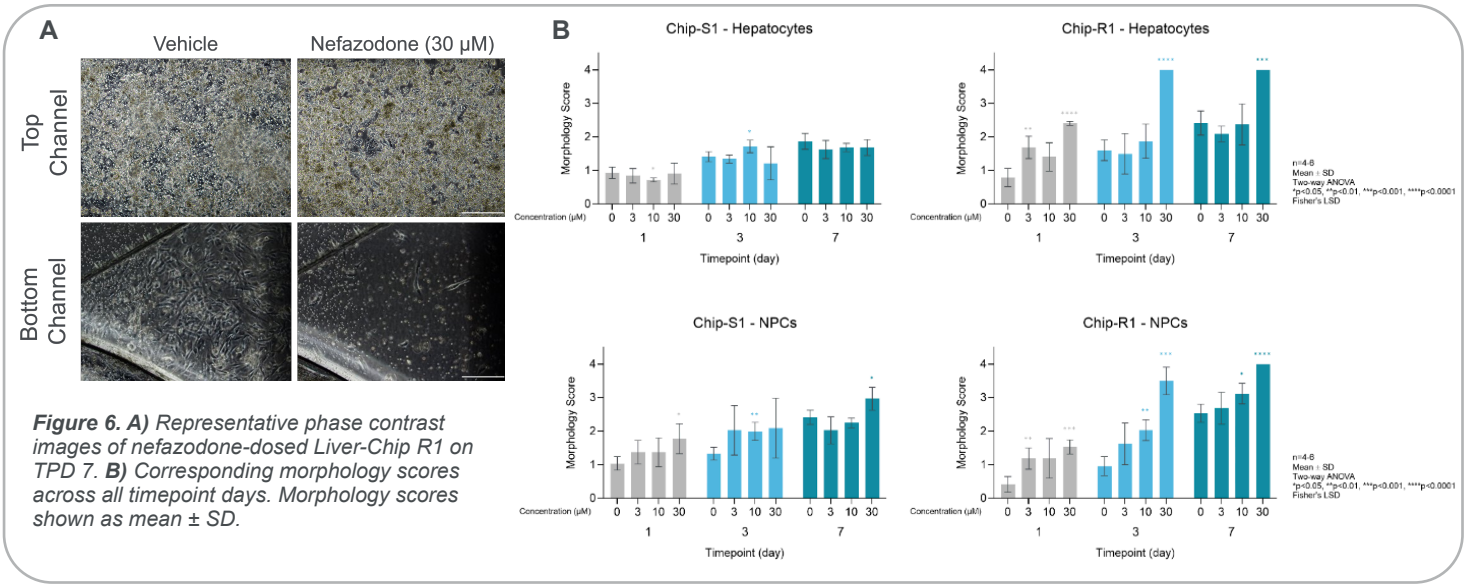

Figure 6. A) *Representative phase contrast images of nefazodone-dosed Liver-Chip R1 on TPD 7.* ***B)*** *Corresponding morphology scores across all timepoint days. Morphology scores shown as mean ± SD.*

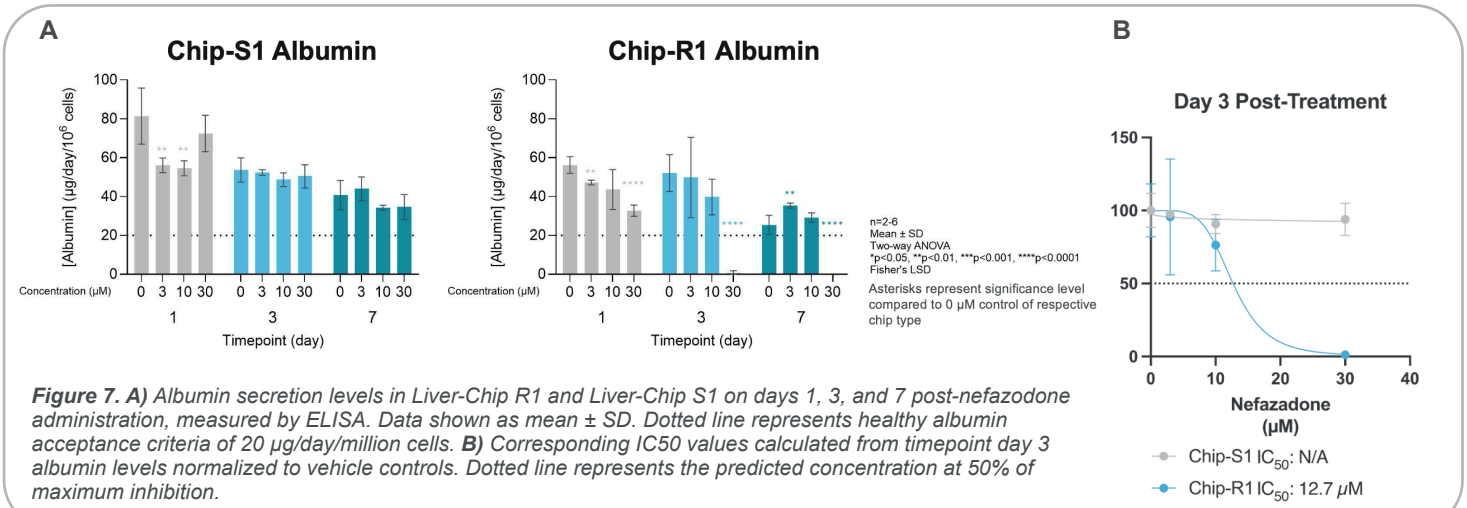

Figure 7. A) *Albumin secretion levels in Liver-Chip R1 and Liver-Chip S1 on days 1, 3, and 7 post-nefazodone administration, measured by ELISA. Data shown as mean ± SD. Dotted line represents healthy albumin acceptance criteria of 20 µg/day/million cells.* ***B)*** *Corresponding IC50 values calculated from timepoint day 3 albumin levels normalized to vehicle controls. Dotted line represents the predicted concentration at 50% of maximum inhibition.*

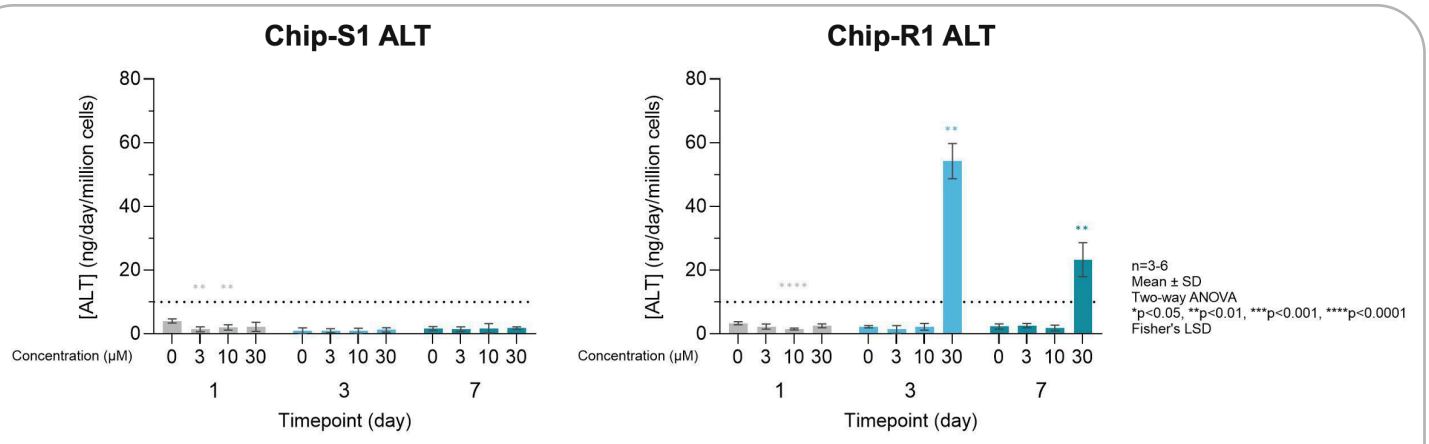

Figure 8. *ALT synthesis levels in Liver-Chip R1 and Liver-Chip S1 on days 1, 3, and 7 post-nefazodone administration, measured by ELISA. Data shown as mean ± SD. ALT levels above 10 ng/day/million cells were considered indicative of liver toxicity.*

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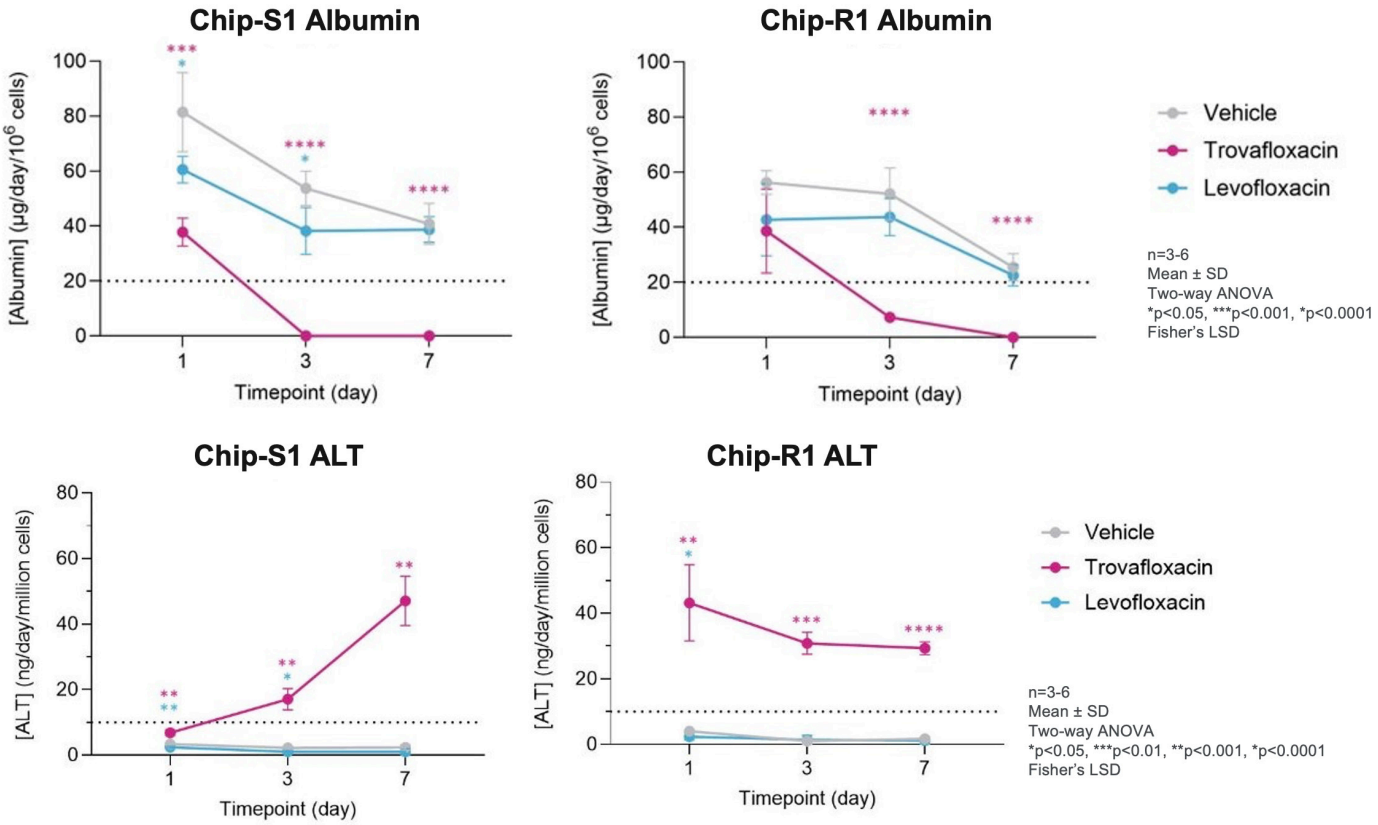

Figure 9. *Albumin synthesis and ALT release levels on Liver-Chip R1 and Liver-Chip S1 treated with trovafloxacin and levofloxacin on days 1, 3, and 7 post-administration. Data shown as mean ± SD.*

Chip-R1 Drug Absorption Characterization

To characterize the ability of the Chip-R1 to minimize drug absorption, a panel of eight drugs was tested on both the Chip-R1 and Chip-S1. These drugs were selected for their relevance to hepatotoxicity or liver metabolic functions, as well as for their broad range of physicochemical properties (see **Table 2**).

The drugs were administered to both channels of acellular Chip-S1 and Chip-R1 consumables at a flow rate of 30 µL/h. To assess compound recovery, effluent was collected from Pod reservoirs (inlets and outlets) for the 24–28 hour exposure interval. Drug recovery rates were calculated as the average ratio of Pod outlet concentration to Pod inlet concentration during this four-hour interval (see **Figure 10**).

Out of the eight drugs tested, three demonstrated high absorption on Chip-S1: nefazodone, bufuralol, and midazolam. These drugs would be predicted to have a significant risk of PDMS absorption based on their relatively high hydrophobicity, low molecular weight, and/or low topological polar surface area. In contrast, Chip-R1 showed a significantly improved recovery profile compared to Chip-S1:

- Bufuralol improved from 60% to 93% recovery
- Midazolam improved from 25% to 85% recovery
- Nefazodone improved from 2.7% to 68% recovery

The remaining drugs tested demonstrated no significant absorption on either Chip-S1 or Chip-R1.

To gain further insight into drug absorption kinetics, a 48-hour time-course analysis was performed using nefazodone, the drug with the lowest recovery. Over the 48-hour period, Chip-S1 demonstrated negligible recovery of nefazodone, while Chip-R1 showed low recovery rates in the initial hours of dosing, which increased to over 80% on average across both top and bottom channels by the end of the 48-hour period. Together, these findings demonstrate the utility of the Chip-R1 in ADME and toxicology applications, even for drugs that are highly prone to absorption into PDMS, such as nefazodone.

Compound	Dosing concentration	logP	Molecular Weight (Da)	Topological Polar Surface Area (Å2)	pKa	Ionization status at pH 7.4
Nefazodone	30 µM	4.3 - 4.7	470	51.6	7.09	Neutral
Diclofenac sodium	20 µM	4.26 - 4.75	318.1	52.2	4	Anionic
Bufuralol HCl	10 µM	2.99 - 3.24	297.82	45.4	13.06	Cationic
Midazolam	20 µM	2.73	325.8	30.2	5.5	Neutral
Labetalol	137 µM	2.7 - 3.1	328.4	95.58	9.3	Cationic
S-mephenyt-oin	35 µM	1.64 - 1.69	218.3	49.4	8.1	Neutral
Phenacetin	30 µM	1.41 - 1.62	179.2	38.3	14.98	Neutral
Trovafloxacin	200 µM	0.3 - 0.9	416.4	99.8	5.9	Zwitterion

Table 2. *Compounds assessed for absorption in acellular Chip-S1 and Chip-R1.*

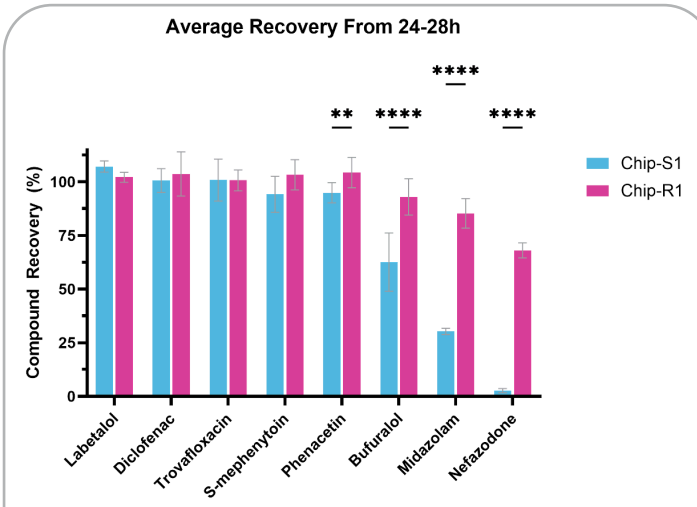

Figure 10. *Average compound recovery between 24–28 hours of flow. Top and bottom channel datapoints were combined for analysis. Data shown as mean ± SD. Significance in recovery determined by two-way ANOVA with Fisher's LSD multiple comparison test. n=3-4 chips per condition, with values for top and bottom channels averaged per chip, **p<0.01, ****p<0.0001*

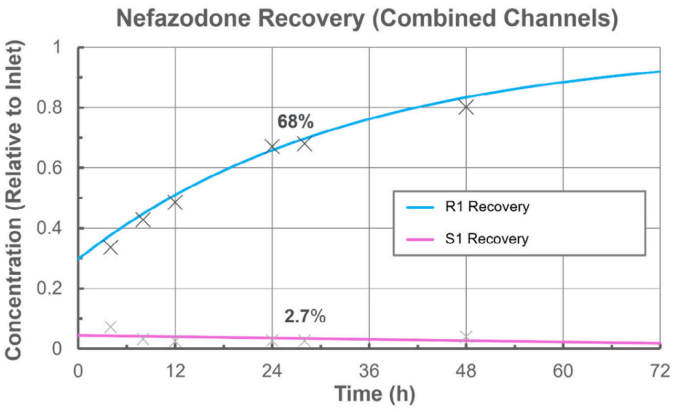

Figure 11. *Absorption kinetics of nefazodone on Chip-R1 versus Chip-S1. Marked X's denote measured data averaged over a four-hour time interval. Solid lines represent the curve fit for instantaneous recovery values. n=3-4 chips per condition.*

Conclusion

The Chip-R1 Rigid Chip offers several advantages over the Chip-S1 Stretchable Chip, such as low drug-absorbing materials, a thinner, pre-activated membrane with smaller diameter pores, and a reduced vascular channel height which allows for an increase in maximum shear stress exposure. These enhancements make the Chip-R1 (and associated Pod-2) an ideal platform for studying small-molecule drug interactions with tissues that do not require stretch.

Results from the above equivalency study show that the Liver-Chip R1 replicates critical aspects of human liver physiology and serves as a biologically relevant model for assessing DILI. Comparative studies show that the Liver-Chip R1 maintains essential liver functionality, such as albumin production and metabolic activity, while improving sensitivity to hepatotoxic compounds prone to PDMS absorption. These findings suggest that the Liver-Chip R1 provides a more suitable model for evaluating the DILI risk of lipophilic small molecules.

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