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Characterization of the Emulate Liver Chip Microphysiological System

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RESEARCH AND TECHNOLOGY DIRECTORATE

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Organ-on-a-chip technologies, otherwise known as microphysiological systems, have been gaining popularity as models that are highly predictive measures of human outcomes for a particular drug or compound. In this report, we have adopted the technology created by Emulate (Boston, MA) to be part of the U.S. Army Combat Capabilities Development Command Chemical Biological Center's (Aberdeen Proving Ground, MD) predictive toxicology program. The Emulate human liver chip provides the Center a unique means of achieving physiologically relevant liver data without the immediate need for an animal model. We can quickly and easily generate tissue effluent data and obtain cellular images of the entire liver environment. Importantly, this technology is amendable to different organs, including the lung, brain, and gut. Here, we validate the liver chip system using the drug methotrexate, which develops a well characterized liver phenotype. The hope moving forward is that the use of organoids created in physiologically relevant ex vivo platforms, such as the Emulate liver chip, will enhance the Department of Defense's analytical capabilities for rapid threat assessment.

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PREFACE

The work described in this report was authorized under Defense Threat Reduction Agency (Fort Belvoir, VA) project number CB10735 and Defense Advanced Research Projects Agency (Arlington, VA) Rapid Threat Assessment project number BQ5066. The work was started in August 2019 and completed in January 2020.

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This report has been approved for public release.

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EXECUTIVE SUMMARY

The work described in this technical report showcases the utility of the Emulate (Boston, MA) organs-on-chips platforms, which combine microscale engineering with cultured living human cells to recreate the physiological and mechanical microenvironment of whole living organs. Emulate scientists have developed their system to accommodate a number of different organs, and here we chose to validate their liver-chip system using the drug methotrexate. Methotrexate overexposure develops a well characterized liver phenotype, which was recapitulated using the chips. This report indicates that the use of organoids created in physiologically relevant ex vivo platforms, such as the Emulate liver chip, will enhance the Department of Defense's analytical capabilities for rapid threat assessment.

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(U) CONTENTS

FIGURES

TABLES

CHARACTERIZATION OF THE EMULATE LIVER CHIP MICRO-PHYSIOLOGICAL SYSTEM

1. INTRODUCTION

1.1 Emulate Liver Chip

Interrogating the effects of a drug or compound on the liver is central to most metabolism, mechanism of action, and toxicity studies. As such, having a reliable liver model capable of predicting metabolic and toxicologic consequences in humans is of paramount importance. Traditionally, researchers have relied heavily on two-dimensional tissue cultures and animal models to make these predictions.[1,2] However, in vivo work can require an exorbitant amount of time and money, and tissue cultures grown on dishes and plates often lack physiological relevancy. For these reasons, microphysiological systems (MPSs) have been gaining traction as more accurate and potentially more cost effective means for predicting human outcomes in response to exposure to drugs or compounds of interest.[3,4] By providing a three-dimensional architecture composed of a multi-cell type microenvironment complete with fluid flow and other cellular and environmental dynamics present in a functional organ, MPSs are leading the charge for in vivo toxicity testing alternatives.[5]

Originally conceptualized at the Wyss Institute for Biologically Inspired Engineering at Harvard University (Cambridge, MA), the Emulate Organs-on-Chips technology (Emulate; Boston, MA) has been applied to multiple organs and biological systems.[4–9] The liver chip is Emulate's hallmark MPS, but the organization also commercially provides a duodenum intestine chip,[8] and a kidney proximal tubule chip.[6] A number of other Organs-on-Chips systems are in varying stages of development, including a lung chip, a cardiac chip, a thrombosis chip, a blood brain barrier chip, and a skin chip. All of Emulate's Organs-on-Chips systems consist of a number of specialized components (Figure 1). The S1 chips are made of polydimethylsiloxane, and are composed of a top and bottom channel seperated by a porous membrane. The chips are housed in Pod portable modules (Emulate), which also function to supply medium to the various cell types. Cells are dosed with drugs through the inlet resevoirs of the Pod portable module, and effluent is collected from the outlet reservoirs for analysis. The flow rate of the medium through the top and bottom channels of the chip is controlled by the Zoë culture module (Emulate). The Zoë culture module accomodates a pump manifold, which is engaged with the chips housed in the Pod portable modules to produce the medium flow. All of the gas exchange within the chips is controlled by the Orb hub module (Emulate).

The top channel of the liver chip houses the human hepatocytes, whereas the bottom layer is seeded with liver sinusoidal endothelial cells (LSECs), human stellate cells (HSCs), and Kupffer cells. One of the benefits to Emulate's two channel chip design is that the multiple cell types can interact with each other in their microenviornment the same way they would in a human liver, with liver specific cytoarchitecture and funtion maintained.[4] The microfluidic component of the chips and the pump system designed to control the flow of medium through the chip and over the cells also lend to the physiological relevancy of the system. Constant flow at the appropriate rate creates the same amount of shear stress the tissues would experience in a human liver. All of these factors allow for a relevant response to drug or compound exposure that is comparable to what is observed in vivo. Because the Emulate liver

chip mimics a human liver physiologically, researchers can perform the same analyses and assess the same liver endpoints as they would in vivo. Liver chip applications optimized for the system by Emulate include, but are not limited to, assessing hepatotoxicity, changes in morphology, oxidative stress, the immune response, metabolism, transcription, and protein production.

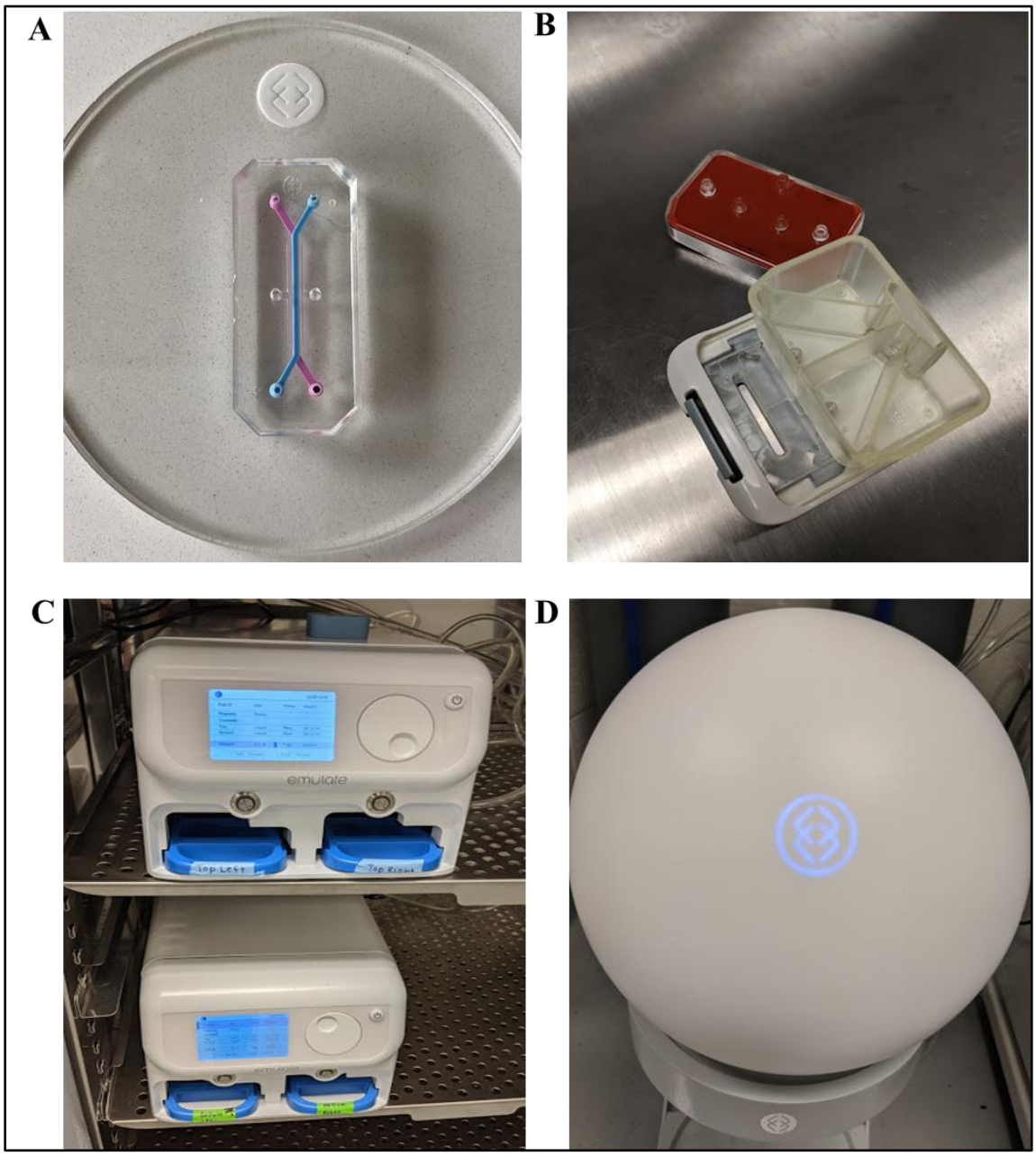

Figure 1. Components of the Emulate MPS. The S1 chip (A) consists of a top channel (blue) and a bottom channel (pink) where the various cells are seeded. The chips are housed in the Pod portable module (B), which contains inlet and outlet reservoirs for medium. The Pod modules are placed in the Zoë culture module (C), which controls flow. Gas exchange is controlled by the Orb hub module (D).

1.2 Methotrexate (MTX) Induced Non-Alcoholic Fatty Liver Disease (NAFLD)/Non-Alcoholic Steatohepatitis (NASH)

Liver disease associated with the accumulation of fat within the liver (not associated with alcohol use) is divided into two types: NAFLD or NASH.[10–12] The former (NAFLD) is defined as the accumulation of fat within the liver not associated with further liver damage. NASH is a more serious condition that includes not only the accumulation of fat within the liver, but also hepatic inflammation and liver cell damage. The long-term effects of this disease can lead to more serious conditions, including hepatic fibrosis, cirrhosis, and the development of hepatocellular carcinoma.[10-12] These conditions can be caused by a variety of factors, but most commonly metabolic syndrome or exposure to specific hepatic-acting drugs.[10–12] Traditionally, this condition is modeled in the laboratory using various animal models. These in vivo models include feeding standard laboratory mice a high-fat diet for an extended period of time or using genetically-modified mice that are prone to obesity.[13] In vivo models take time to develop and have the typical problem of appropriately correlating the results to human disease. NAFLD/NASH can also be studied using in vitro hepatic models.[14] In vitro hepatic models involve examining hepatic cell lines or primary hepatic cells in culture. These models can produce rapid results, but immortalized hepatic cell lines do not accurately represent in vivo hepatocytes, and primary hepatocytes have a limited ability to retain viability in a typical two-dimensional culture. Recently, researchers have developed the ability to culture hepatocytes in suspended spheroids in a three-dimensional architecture. These models are closer to in vivo models than traditional two-dimensional cultures but still lack all of the cells found in the liver and the flow associated with the vasculature. The Emulate liver chip provides a unique opportunity to model NAFLD/NASH in vitro because this model contains four different liver-specific cells (primary hepatocytes, LSECs, HSCs, and Kupffer cells).[4]

Clinical presentation of NAFLD/NASH has been observed following MTX treatment, as either a chemotherapeutic agent or immune system suppressant. The chemical structure of MTX is shown in Figure 2. MTX's primary mechanism of action is interrupting the metabolism of folic acid; this process is essential for the synthesis of DNA. One of the more common side effects of MTX administration is hepatotoxicity, which can lead to NAFLD/NASH through the accumulation of lipids within the liver.[15,16] In this report, we examine how MTX treatment of the Emulate liver chip recapitulates the hepatic steatosis associated with NAFLD/NASH.

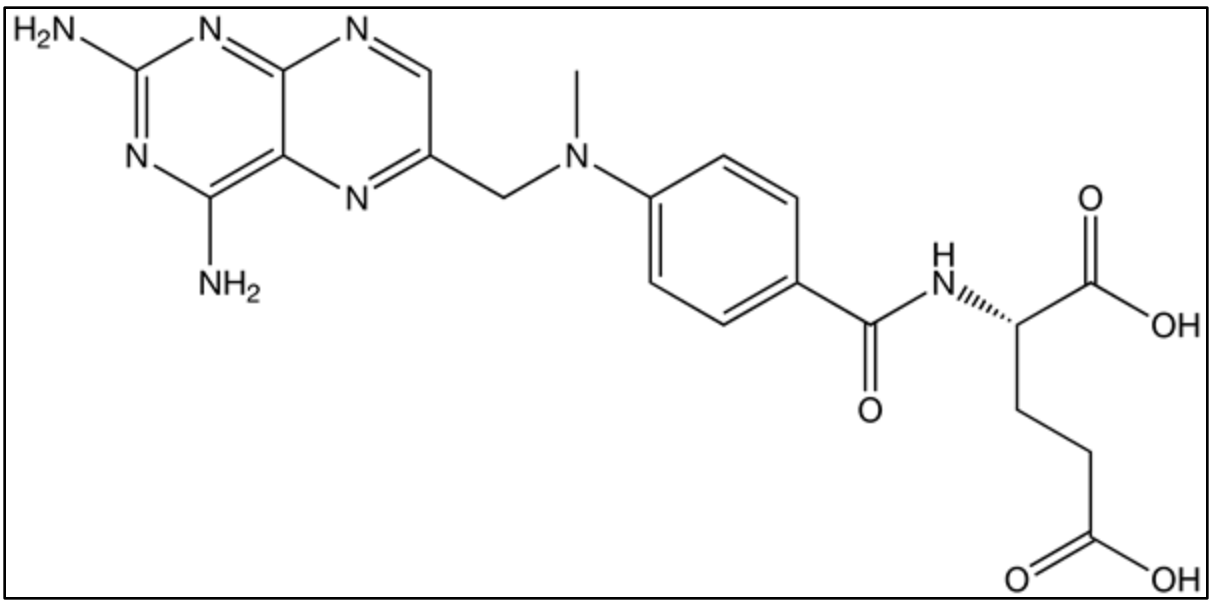

Figure 2. Chemical structure of MTX.

1.3 Aims of this Study

There is a great deal of interest in expanding the U.S. Army Combat Capabilities Development Command Chemical Biological Center's (DEVCOM CBC; Aberdeen Proving Ground, MD) suite of predictive toxicology tools. In this study, we sought to determine whether the Emulate liver chip system would be a viable means for assessing the toxicity of various compounds of interest. First, we wanted to ensure that the system was fully functional in our laboratory space. The Emulate liver chip system consists of a complex liver microenvironment, and creating and maintaining the tissues requires weeks of in-depth technical training. After we verified that we had viable liver chips, we set out to test the ability of the liver chip system to recapitulate a complex hepatotoxicity phenotype (i.e., NAFLD) following chemical exposure.

2. METHODOLOGY

2.1 Chemicals

Table 1 lists the materials and chemicals used in this study together with the manufacturers from which they were purchased.

Table 1. Materials Used

Material/Chemical	Manufacturer
Liver Bio-Kit*	Emulate
DPBS (-/-) (without Ca^{++}, Mg^{++})	Corning; Corning, NY
10× DPBS (-/-) (without Ca^{++}, Mg^{++})	
Trypan blue	Millipore Sigma; Burlington, MA
Percoll solution	
Trypsin-EDTA	
WEM + phenol red	
WEM - phenol red	
CSC medium kit†	Cell Systems; Kirkland, WA
Matrigel	Corning
Fibronectin	Thermo Fisher Scientific; Waltham, MA
Collagen type I	Corning
Penicillin-streptomycin	Millipore Sigma
L-GlutaMax	Thermo Fisher
L-ascorbic acid	Millipore Sigma
Dexamethasone	
FBS	
ITS +	Corning
PFA	Millipore Sigma
Human albumin ELISA kit	Abcam; Cambridge, UK
CyQUANT LDH cytotoxicity assay	Thermo Fisher
Human TNF-alpha Quantikine ELISA kit	R&D Systems; Minneapolis, MN
Saponin	Millipore Sigma
Goat serum	Thermo Fisher
LipidSpot lipid droplet stain	Biotium; Fremont, CA
Rabbit anti-αSMA antibody	Abcam
Mouse anti-CD68 antibody	
Goat anti-rabbit IgG H&L Texas red	
Goat anti-mouse IgG H&L Alexa fluor 488	
Hoechst nuclear counterstain	Molecular Probes; Eugene, OR

*The kit includes 24 S1 chips, human hepatocytes, human HSCs, human Kupffer cells, human LSECs, ER-1 solution, and ER-2 solution.
†The kit includes CSC medium, culture boost, attachment factor, and cell freezing medium.
αSMA, alpha smooth muscle actin; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; H&L, heavy and light; IgG, immunoglobulin G; ITS, insulin, transferrin, and selenous acid; LDH, lactate dehydrogenase; PFA, paraformaldehyde; TNF, tumor necrosis factor; WEM, William's E medium.

2.2 Cell Storage

Human hepatocytes, HSCs, Kupffer cells, and LSECs were purchased directly from Emulate. The cells were kept frozen in liquid-nitrogen storage (vapor phase) until they were cultured.

2.3 Reagent Preparation

All reagents were aliquoted at appropriate volumes prior to use to avoid multiple freeze-thaw cycles. Fibronectin was resuspended in cell-culture grade water to a concentration of 1 mg/mL. Matrigel was thawed overnight on slushy ice in a 4 ℃ refrigerator. Using cold pipet tips, Matrigel was aliquoted to 5 mg based on the specific stock concentration. L-ascorbic acid was resuspended in cell-culture grade water to a concentration of 50 mg/mL. Dexamethasone was resuspended in cell-culture grade dimethyl sulfoxide (DMSO) at concentrations of 1 and 10 mM. All reagent aliquots were stored at –20 ℃. MTX stock solutions were prepared by dissolving the compound in cell culture grade DMSO at a concentration of 30 mM.

Media preparations used in this study are presented in Table 2.

Table 2. Media Preparations

Medium		**Component**	
Name	**Volume (mL)**	**Name**	**Volume**
Base LSEC culture medium	500	CSC basal medium	485 mL
		Culture boost	10 mL
		Penicillin/streptomycin	5 mL
Complete LSEC culture medium	50	Base LSEC culture medium	45 mL
		FBS	5 mL
Base hepatocyte seeding medium	500	WEM + (with phenol red)	490 mL
		Penicillin/streptomycin	5 mL
		L-GlutaMax	5 mL
Complete hepatocyte seeding medium	200	Base hepatocyte seeding medium	187.78 mL
		ITS+ premix	2 mL
		Ascorbic acid	200 µL
		Dexamethasone (10 mM)	20 µL
		FBS	10 mL
Base hepatocyte maintenance medium	500	WEM (without phenol red)	490 mL
		Penicillin/streptomycin	5 mL
		L-GlutaMax	5 mL
Complete hepatocyte maintenance medium	50	Base hepatocyte maintenance medium	49.445 mL
		ITS+ premix	500 µL
		Ascorbic acid	50 µL
		Dexamethasone (1 mM)	5 µL
Hepatocyte overlay medium	20	Complete hepatocyte maintenance medium	19.5 mL
		Matrigel	0.5 mL

continued

Table 2. Media Preparations (continued)

Medium		Component	
Name	**Volume (mL)**	**Name**	**Volume**
NPC seeding medium	50	Complet+e hepatocyte maintenance medium (omitting dexamethasone)	22.5 mL
		Base LSEC culture medium	22.5 mL
		FBS	5 mL
NPC maintenance medium	50	Complete hepatocyte maintenance medium (omitting dexamethasone)	24.5 mL
		Base LSEC culture medium	24.5 mL
		FBS	1 mL

NPC, non-parenchymal cell.

2.4 LSEC culture

LSECs were removed from liquid nitrogen storage and quick thawed by placing the cryovial in a 37 ℃ water bath. The contents of the vial were transferred to a 15 mL conical tube containing 3 mL of warm complete LSEC culture medium. The vial was rinsed once with 1 mL of medium, and the 15 mL conical tube was brought up to a volume of 15 mL of complete LSEC culture medium. The cell solution was centrifuged at 200 × *g* for 5 min at room temperature. The supernatant was aspirated, and the cells were resuspended in 15 mL of fresh complete LSEC culture medium. The LSEC suspension was added to a T-75 flask and incubated at 37 ℃ and 5% CO_2. LSECs were cultured for at least two days before chip seeding, and medium was exchanged every two days.

2.5 Chip Activation

ER-1 and ER-2 reagents are both light sensitive, so all manipulations were performed in a biosafety cabinet (BSC) with the lights turned off. Before preparation of the activation solution, ER-1 and ER-2 were allowed to equilibrate to room temperature. One milliliter of ER-2 was added to the ER-1 vial, and the contents were added directly to a 15 mL conical tube. An additional 1 mL of ER-2 was added to the ER-1 vial to collect remaining material, then this solution was added to the 15 mL conical tube. This process was repeated twice more. A total of 6 mL of ER-2 was added to the 4 mL of ER-1 solution in the 15 mL conical tube for a final concentration of 0.5 mg/mL. Using a P200 pipet, test personnel introduced the ER-1 solution to the top and bottom channels of the S1 chip at volumes of 50 and 20 µL, respectively, being careful not to introduce bubbles. Excess solution was aspirated from the top of the chip. The chips were activated under constant UV light for 20 min. After UV activation, the ER-1 solution was aspirated from both channels, and both channels were washed with 200 µL of ER-2 solution. The top and bottom channels of each chip were washed with 200 µL of 1× DPBS, and cold 1× DBPS was left in the channel. The chips were incubated at 4 ℃ overnight.

2.6 Extracellular Matrix (ECM) Coating

ECM was prepared on ice by combining collagen I and fibronectin in ice-cold 1× DPBS at concentrations of 100 and 25 µg/mL, respectively. After ECM preparation, the cold 1× DPBS was removed from the top and bottom channels of the chips. ECM was added to each channel using a P200 pipet until small droplets formed on the channel outlets. Droplets of ECM were placed on all four ports of each chip, and the chips were incubated at 4 °C overnight.

2.7 Hepatocyte Seeding

The chips were equilibrated to room temperature and washed three times with 200 µL of complete hepatocyte seeding medium. The final wash was left in the top and bottom channels of the chips. Human hepatocytes were removed from liquid nitrogen storage, and the cryovial was quick thawed in a 37 ℃ water bath. The thawed cell solution was quickly added to 3 mL of warm complete hepatocyte seeding medium in a 50 mL conical tube. The vial was rinsed with 1 mL of medium, which was then transferred to the 50 mL conical tube. Complete hepatocyte seeding medium was slowly added to the 50 mL conical tube until the volume was brought to 35 mL. Fifteen milliliters of 90% Percoll solution in 1× DPBS was slowly layered on top of the 35 mL of cell solution. The cells were centrifuged at 96 × *g* for 6 min at room temperature. The majority of the supernatant was carefully aspirated so as not to disturb the pellet, leaving 3–5 mL of supernatant. The pellet was gently resuspended in the remaining supernatant by tilting and rotating the 50 mL conical tube. Complete hepatocyte seeding medium was added to the 50 mL conical tube until the total volume was 50 mL, and the cell solution was centrifuged at 72 × *g* for 4 min at room temperature. The supernatant was carefully removed until only 1–2 mL of supernatant was left, and the pellet was carefully resuspended as described above. The human hepatocytes were counted in trypan blue solution using a hemocytometer (Thermo Fisher) and resuspended to a final cell density of 3.5×10^6 cells/mL in complete hepatocyte seeding medium. Fifty milliliters of the cell suspension was carefully added to the top channel of each chip. The chips were incubated at 37 ℃ with 5% CO_2 for 3 h, or until the hepatocytes had attached. After the hepatocytes were attached, a gravity wash was performed on each chip by gently dropping 200 µL of complete hepatocyte seeding medium on top of both inlet ports of the top and bottom channels. The chips were incubated overnight at 37 ℃ with 5% CO_2.

2.8 Hepatocyte Matrigel Overlay

Matrigel was slowly thawed on slushy ice. Hepatocyte overlay medium was also prepared on ice by diluting the Matrigel into ice-cold complete hepatocyte maintenance medium with cold tips to a concentration of 250 µg/mL. Chips were removed from the incubator and washed with 200 µL of complete hepatocyte maintenance medium. Two hundred microliters of complete hepatocyte overlay medium was pipetted into the top channel of each tip, leaving droplets on both the inlets and the outlets. The chips were incubated overnight at 37 ℃ and 5% CO_2.

2.9 NPC Seeding

The LSECs were harvested from the T-75 using 3 mL of trypsin-EDTA. Nine milliliters of warm NPC seeding medium was added to the cell suspension, and the entire 12 mL of solution was centrifuged in a 15 mL conical tube at 200 × *g* for 5 min. The supernatant was aspirated, leaving 100 µL in the tube with which to resuspend the LSECs. The LSECs were counted in trypan blue solution using a hemocytometer and resuspended to a cell density of 9×10^6 cells/mL in cold NPC seeding medium. The cell suspension was kept on ice while the other cell types were prepared for seeding. HSCs were removed from liquid nitrogen storage, and the cryovial was quick thawed in a 37 ℃ water bath. The thawed cell solution was quickly added to 3 mL of warm NPC seeding medium in a 15 mL conical tube. The vial was rinsed with 1 mL of medium, which was then transferred to the 15 mL conical tube. The cell suspension was increased to 15 mL with NPC seeding medium and centrifuged at 250 × *g* for 5 min. The supernatant was aspirated, leaving 100 µL in the tube with which to resuspend the HSCs. The cells were counted in trypan blue solution using a hemocytometer, resuspended to a cell density of 0.3×10^6 cells/mL in cold NPC seeding medium, and placed on ice. Kupffer cells were removed from liquid nitrogen storage, and the cryovial was quick thawed in a 37 ℃ water bath. The thawed cell solution was quickly added to 3 mL of warm NPC seeding medium in a 15 mL conical tube. The vial was rinsed with 1 mL of medium, which was then transferred to the 15 mL conical tube. The cell suspension was increased to 15 mL with NPC seeding medium and centrifuged at 250 × *g* for 5 min. The supernatant was aspirated, leaving 100 µL in the tube with which to resuspend the Kupffer cells. The cells were counted in trypan blue solution using a hemocytometer and resuspended to a cell density of 1.5×10^6 cells/mL in cold NPC seeding medium. The three NPC cell suspensions were mixed in a 1:1:1 ratio (v/v/v) in a 15 mL conical tube on ice. Twenty microliters of the combined NPC suspension was added to the bottom channel of each chip. After seeding, the chips were inverted and incubated at 37 °C and 5% CO_2 for 4 h, or until the cells attached. After the cells were attached to the bottom channel, a gravity wash was performed with 200 µL of hepatocyte maintenance medium for the top channel and NPC seeding medium for the bottom channel. The chips were incubated overnight at 37 ℃ and 5% CO_2.

2.10 Chips to Pod Portable Modules and Pod Portable Modules to Zoë Culture Module

Complete hepatocyte maintenance medium and NPC maintenance medium was warmed in a 37 ℃ water bath for 1 h. To reduce the risk for bubbles in the chips, the medium used for the Pod portable modules was gas equilibrated in 50 mL steriflip conical tubes for 5 min before use. Three hundred microliters of complete hepatocyte maintenance medium was added directly over the Pod port of the top channel outlet reservoir, and 300 µL of NPC maintenance medium was added to the bottom channel outlet reservoir in the same manner. Three milliliters of the appropriate medium was added to the inlet reservoirs of each Pod portable module. The Pod portable modules were inserted into the Zoë culture module and primed. After the modules were primed, the top and bottom channels of the chips were washed with 200 µL of the appropriate medium and manually connected to each Pod portable module. The Pod portable modules with chips were then placed back into the Zoë culture module, and a regulate cycle was run to stabilize the fluidics of the system. When the cycle was complete, the flow conditions

changed to 30 µL/h for both top and bottom channels. The top and bottom inlet reservoir medium was replenished every two days while the chips were under flow.

2.11 MTX Solution Preparation and Dosing

MTX working solution was prepared by diluting the drug in NPC maintenance medium to a final concentration of 30 µM. A vehicle control was also prepared by diluting cell culture grade DMSO in NPC maintenance medium to a final concentration of 0.1%. The bottom channel inlet reservoir medium was aspirated and replaced with 3 mL of medium containing either MTX or DMSO vehicle control so that the drug would be introduced on the endothelial channel. The Pod portable modules were returned to the Zoë culture module, and the MTX exposure was initiated by increasing the flow rate of the Zoë culture module to 600 µL/h for 5 min. The flow rate was then returned to 30 µL/h. Media containing the drug was replenished in the bottom channel inlet reservoir every 2 days for 10 days total.

2.12 Human Albumin ELISA

The albumin ELISA was performed in accordance with the protocol set out in Abcam's human albumin ELISA assay kit. All endpoint optical densities (ODs) were read on a FlexStation III multimode plate reader (Molecular Devices; San Jose, CA) at a wavelength of 450 nm.

2.13 LDH Cytotoxicity Assay

The LDH cytotoxicity assay was performed in accordance with the protocol set out in Thermo Fisher's CyQUANT LDH cytotoxicity assay. All endpoint ODs were read on a FlexStation III multimode plate reader at wavelengths of 490 and 680 nm.

2.14 Immunostaining

The NPCs were fixed with 4% PFA in 1× DPBS for 15 min at room temperature. Cells were then permeabilized with 1% saponin in 1× DPBS for 30 min at room temperature. Blocking was performed by adding 1% bovine serum albumin and 10% goat serum in 1× DPBS, which was incubated overnight at 4 °C. The primary antibody solution was made by diluting either rabbit anti-αSMA or mouse anti-CD68 1:1000 and 1:100 in blocking buffer, respectively, which was added to the bottom channel of the chips. The chips were incubated overnight at 4 °C. The secondary antibody solution was prepared by diluting Texas red-conjugated goat anti-rabbit IgG H&L or Alexa Fluor 488-conjugated goat anti-mouse IgG H&L 1:500 in blocking buffer. Hoechst counterstain was added to the secondary antibody solution at a 1:10,000 dilution. The secondary antibody solution was added to the bottom channels of the chips, and the chips were incubated for 2 h at room temperature in the dark. The chips were washed three times with 1× DPBS, and images were obtained using a BZ-X fluorescence microscope (Keyence; Itasca, IL).

2.15 Lipid Staining

The NPC cells were treated with a combination of the LipidSpot lipid droplet stain and Hoechst counterstain in blocking buffer at a dilution of 1:10,000 for 15 min at room temperature. The chips were washed three times with 1× DPBS, and images were obtained using a Keyence BZ-X Fluorescence Microscope.

3. RESULTS

3.1 Liver Chip Function and Viability

Emulate has previously used albumin production to verify replication of hepatocyte phenotype in vitro.[4] Effluent collected from the outlet reservoir of the Pod portable modules was collected 4 and 14 days after the Zoë culture module was connected. By day 14, the chips had been treated for 10 days with either DMSO or MTX. Hepatocyte albumin production was observed to be within an acceptable range compared with previous studies performed by Emulate (Figure 3). A significant decrease in albumin was observed in MTX-treated chips by day 14 after the Zoë culture module was connected. Consistently less albumin was detected in effluent collected from the bottom channel, as expected.

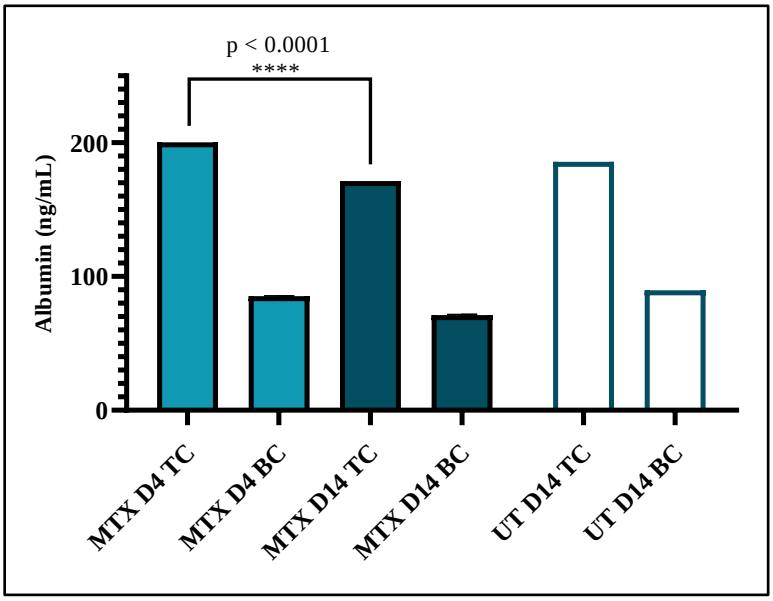

Figure 3. Human albumin ELISA performed on liver chip effluent from the top channel (TC) and bottom channel (BC) collected on day 4 (D4) and day 14 (D14) post Zoë connection. MTX or DMSO vehicle control (UT) exposure began on D4. Each point represents the average albumin production of three individual chips, with the error bars indicating standard deviations. The concentrations were determined by standard curve. Probability (*P*) values were determined by unpaired T-test with a Welch's correction.

LDH has been used as a marker of cell death and toxicity because the enzyme is released into the culture media following necrosis/apoptosis. Therefore, LDH release was used to determine whether MTX had any cytotoxic effects on the different cell populations within the liver chips. LDH release was not detected in effluent collected from the outlet reservoirs of either the top or bottom channels (Table 3). This indicates that all of the cell populations maintained viability throughout the course of the experiment, and that 10 days of MTX exposure at the concentration tested does not result in cell death.

Table 3. Liver Chip Cytotoxicity

Test Condition	**Day**	**Channel**	**% Cytotoxicity Compared to Positive Control**
Untreated	1	TC	1.6
Untreated	1	BC	4.7
MTX	1	TC	0.48
MTX	1	BC	2.8
Untreated	10	TC	0.37
Untreated	10	BC	2.36
MTX	10	TC	0.33
MTX	10	BC	3.3

The presence of viable Kupffer cells was also assessed by immunostaining with an antibody against the commonly used macrophage marker, CD68. Kupffer cells were visualized within the bottom channel of the chips as shown in Figure 4. Nonspecific antibody binding also allowed us to visualize some of the surrounding LSECs.

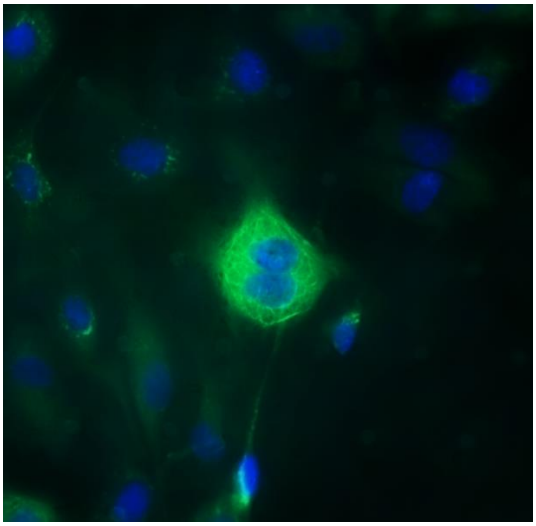

Figure 4. Anti-CD68 Alexa Fluor 488 immunostain of a macrophage (green) present in the bottom channel cell layer. Surrounding cells are LSECs. Nuclei (blue) are counterstained with Hoechst nuclear counterstain.

3.2 Liver Chip MTX Phenotype Assessment

NAFLD and NASH are common outcomes of prolonged MTX exposure, or even acute exposure at high doses. Included in the number of phenotypes associated with this disease are steatosis in hepatocytes and NPCs, HSC activation (which leads to fibrosis), and inflammation. Here we set out to determine whether these adverse side effects associated with MTX exposure could be observed in the liver chips.

After 10 days of MTX exposure, the hepatocyte layer of the liver chips was observed using brightfield microscopy (Figure 5). Lipid droplets were readily visible in all of the treated hepatocytes, whereas the hepatocytes that received the DMSO vehicle control were mostly devoid of anything representing lipid accumulation. A lipid droplet stain was also performed on the bottom channel of the liver chip, as lipid accumulation could not be visualized using brightfield microscopy (Figure 6). In accordance with the MTX-exposed hepatocytes, more lipids were observed in the MTX-treated NPC layer than the NPC layer treated with the DMSO vehicle control. However, it is unclear if these lipids are accumulating within cells.

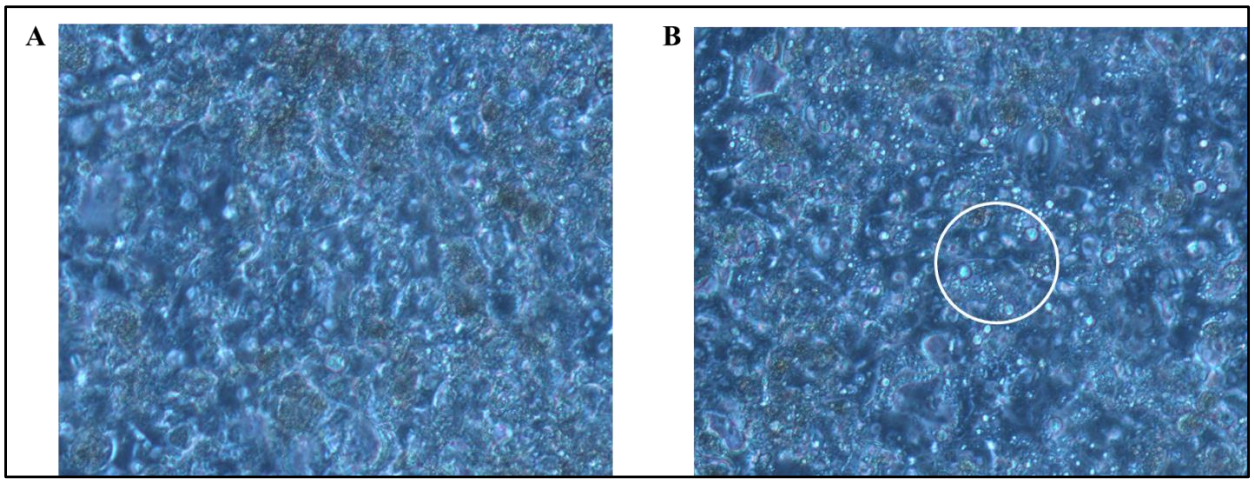

Figure 5. Brightfield images of hepatocytes after a 10 day exposure to (A) DMSO vehicle control, or (B) MTX. Examples of lipid droplets formed in the MTX treated cells can be seen in the white circle.

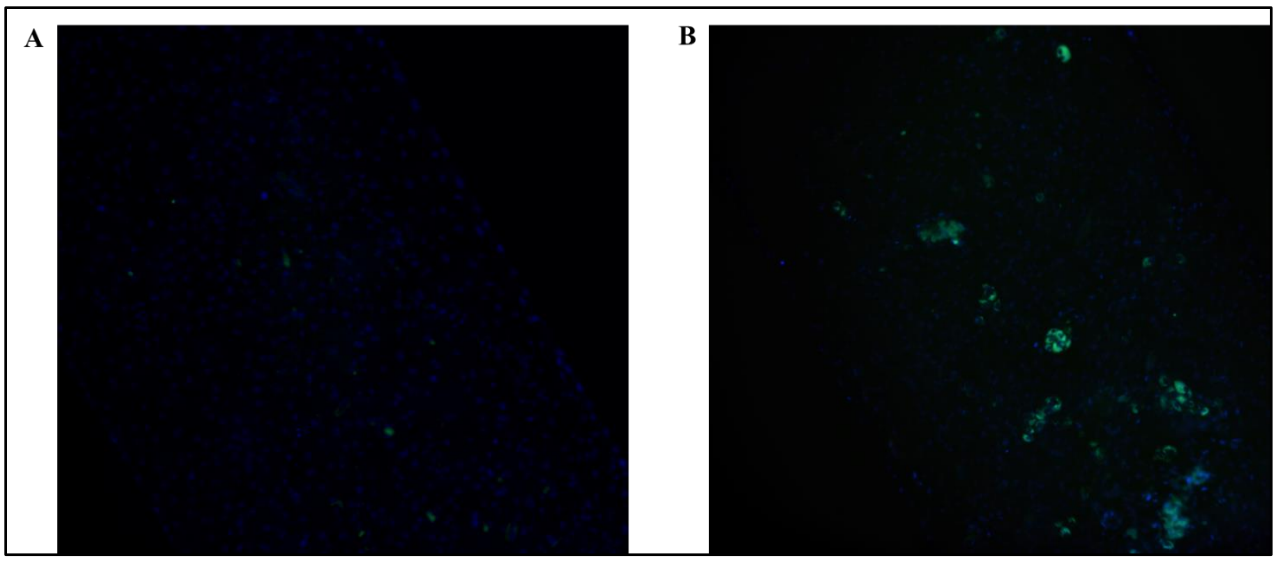

Figure 6. Lipid droplet stain of NPCs treated for 10 days with (A) DMSO vehicle control, or (B) MTX. Lipid accumulation is shown in green. Nuclei (blue) are counterstained with Hoechst nuclear counterstain.

Smooth muscle actin is used as a representative marker of fibrosis and stellate cell activation. We used an anti-αSMA immunostain to look for HSCs and HSC activation in the NPC layer of the liver chips in response to MTX exposure. In comparison with the DMSO vehicle control chips, far more αSMA was observed in the MTX-treated chips (Figure 7). In addition to an overall increase in protein, it also looks as though more HSCs are present in the MTX-treated chips due to HSC activation.

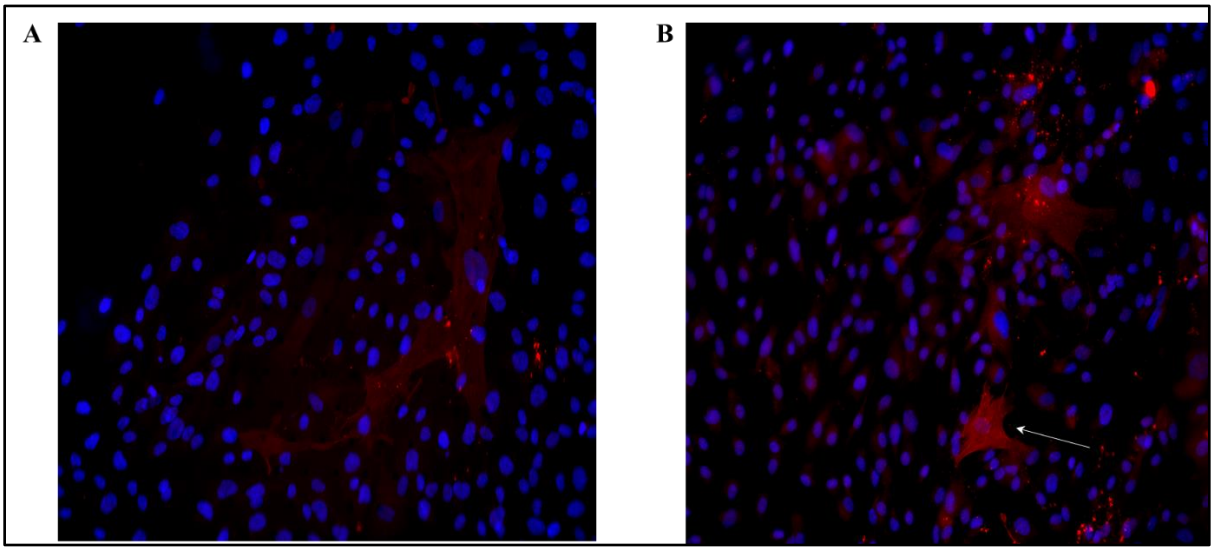

Figure 7. Anti-αSMA Texas red immunostain of HSCs (arrow) present in the bottom channel cell layer of chips treated for 10 days with (A) DMSO vehicle control, or (B) MTX. Nuclei (blue) were counterstained with Hoechst nuclear counterstain.

4. DISCUSSION

Traditionally, mode of action and toxicity studies have relied heavily on two-dimensional tissue cultures on plastic plates and dishes and animal models. However, there are major challenges and limitations associated with both model systems. Two-dimensional cultures usually lack physiological relevance compared with in vivo models, and animal studies are often costly and time-consuming for researchers. Organ-on-a-chip technologies have been gaining popularity as better predictive models for assessing the human effect of various drugs and compounds. In this study, we adequately highlighted the utility of the Emulate liver chip system through characterization of the well-studied drug MTX. We presented a few potential ways this technology could be applied as a predictive toxicological tool to accurately assess the effects of any compound of interest before requiring an animal model. Although the Emulate organ chips do increase researchers' ability to perform more significant in vitro work, there may be limitations to the system.

MPS systems allow for more high-content and human-relevant testing and analysis due to their small laboratory footprint and general ease of use. However, the main highlight of the Emulate system is how closely the microenvironment created in the chip resembles a viable human liver, both physically and functionally. We were successful in detecting all four cell types used in the system through immunostaining and brightfield microscopy, and cytotoxicity assays suggested that the majority of the cells housed within the chips were viable. This viability was sustained after MTX treatment, which corroborates in vivo data that suggests MTX is not cytotoxic at the concentrations tested.[17] Along with the cellular arrangement observed in microscopy images and the viability analysis, the albumin ELISA results generated in this study were a testament to hepatic function, as the concentrations detected in effluent were close to what is produced in vivo, as well as in previous studies conducted by Emulate.[4] The level of albumin secretion detected was also reproducible from chip to chip.

A major goal of this study was to determine whether liver chips could accurately and consistently mimic the characteristics of an in vivo liver, specifically in this case in response to MTX exposure. Lipid droplets were observed inside MTX-treated hepatocytes under brightfield microscopy. This condition, which is known as steatosis, is one of the early indicators of NAFLD and NASH. Fat accumulation in hepatocytes results in the induction of fibrogenic activation of HSCs.[18] When activated, HSCs become proliferative, fibrogenic myofibroblasts that produce an ECM, that is, liver fibrosis.[18,19] The primary marker for HSC activation is αSMA.[20] The increase in αSMA observed in MTX-treated cells in Figure 6 is likely due to induced HSC activation as a result of hepatocyte steatosis occurring in the top layer of the lipid chip. An overall increase in number of stellate cells within the MTX-treated chip was also observed, a further indication that the Emulate liver chip system is capable of generating disease phenotypes.

An additional byproduct of HSC activation is the release of internal lipids, such as retinyl acids and triglycerides,[21] which would likely occur after HSC activation due to prolonged exposure to MTX. However, the lipid droplet stain performed on the NPC layer of liver chips in this study identified more lipid buildup in the MTX-treated chips. It's possible that these lipids

are aggregating outside the cell, which would attribute to the lack of lipids detected in the bottom layer of the untreated chips, thus aligning still with the overall fibrotic phenotype.

Although this study found the Emulate liver chip to be an appropriate tool for the characterization of some primary disease states, using the chips for further toxicological and molecular assessments may be challenging, primarily due to the limited number of cells per chip. There are roughly 175,000 hepatocytes, 60,000 LSECs, 10,000 HSCs, and 2,000 Kupffer cells collectively seeded within the top and bottom channels. This means that cells from multiple chips would likely need to be pooled together to perform proteomic or metabolomic analysis. If this is the case, the system that Emulate has designed is no longer high-content. Moreover, if multiple chips are required per replicate or time point, the overall cost will be higher, especially compared with two-dimensional tissue culture analysis, and likely even animal models. Future studies will aim to extract protein, RNA, and cellular metabolites to determine the feasibility of these types of analyses using cells from Emulate chip systems. For now, Emulate's chip systems have proven to be a useful tool for visualizing and quantifying phenotypic characteristics associated with exposure to compounds of interest.

5. CONCLUSIONS

This study (sponsored by the Defense Threat Reduction Agency and the Defense Advanced Research Projects Agency's Rapid Threat Assessment Program) provides insight into the utility of MPSs, specifically the Emulate human liver chip. The collection of in vitro assays and techniques implemented in this study on both tissue culture effluent and the multiple cell types within the chip can be applied to any compound to assess liver toxicity before an animal model is needed and will serve as part of our predictive toxicological assessment program. The addition of the Emulate system will help DEVCOM CBC researchers characterize compounds phenotypically and mechanistically, and ultimately will provide useful information that can be applied to medical countermeasure development.

We have concluded that the Emulate liver chip will serve as a useful surrogate to any in vivo model given how closely the data collected in this study match the effects of the well-studied drug MTX in humans. The next logical step is to apply Emulate's human chip systems to chemical weapons agents (CWAs), compounds of interest, and lesser-studied compounds with the goal of collecting novel phenotypic and mechanistic data. With the additional work proposed for the liver chip comes the desire to expand to other organs, especially the lungs and the skin. Inhalation, or inhalation with dermal exposure, is the primary concern to the warfighter with regard to CWAs. A dermal chip could be a valuable addition to the repertoire of organ-chip systems Emulate is currently developing. Emulate has developed lung-chip technology but has yet to establish mechanisms for aerosol delivery to the chips. Traditionally, inhalation studies are performed using animal models, so a lung chip with this capability would be indispensable to the chemical defense research field.

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ACRONYMS AND ABBREVIATIONS

αSMA	alpha smooth muscle actin
BC	bottom channel
BSC	biosafety cabinet
CWA	chemical weapons agent
D	day
DEVCOM CBC	U.S. Army Combat Capabilities Development Command Chemical Biological Center
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
H&L	heavy and light
HSC	human stellate cell
IgG	immunoglobulin G
ITS	insulin, transferrin, and selenous acid
LDH	lactate dehydrogenase
LSEC	liver sinusoidal endothelial cells
MPS	microphysiological system
MTX	methotrexate
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NPC	non-parenchymal cell
OD	optical density
P	probability
PFA	paraformaldehyde
TC	top channel
TNF	tumor necrosis factor
UT	vehicle control
WEM	William's E medium

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