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Liver tissue engineering in the evaluation of drug safety

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Abstract

Assessment of drug–liver interactions is an integral part of predicting the safety profile of new drugs. Existing model systems range from *in vitro* cell culture models to FDA-mandated animal tests. Data from these models often fail, however, to predict human liver toxicity, resulting in costly failures of clinical trials. *In vitro* screens based on cultured hepatocytes are now commonly used in early stages of development, but many toxic responses in vivo seem to be mediated by a complex interplay among several different cell types. We discuss some of the evolving trends in liver cell culture systems applied to drug safety assessment and describe an experimental model that captures complex liver physiology through incorporation of heterotypic cell–cell interactions, 3D architecture and perfused flow. We demonstrate how heterotypic interactions in this system can be manipulated to recreate an inflammatory environment and apply the model to test compounds that potentially exhibit idiosyncratic drug toxicity. Finally, we provide a perspective on how the range of existing and emerging *in vitro* liver culture approaches, from simple to complex, might serve needs across the range of stages in drug discovery and development, including applications in molecular therapeutics.

Keywords

3D; clearance; co-culture; flow; hepatocyte; idiosyncratic; in vitro; inflammation; Kupffer; lipopolysaccharide; liver; metabolism; model; ranitidine; safety; tissue engineering; toxicity

1. Introduction

Liver tissue engineering is driven by disparate forces on two separate fronts: large (organ)-scale models aimed at replacement of function in patients of liver failure and small scale

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laboratory systems for the purpose of studying drug metabolism, toxicity, hepatocellular function and liver disease [1]. The success of both types of models hinges on maintenance of liver function *in vitro*. This paper focuses on emerging *in vitro* liver models used for testing drug safety, an application that despite our considerable understanding of fundamental liver biology remains challenging in the development of safe, therapeutic drug candidates. In this context, the criteria for defining 'maintenance of liver function' *in vitro* are arguably growing, from criteria classically focused on preservation of hepatocyte Phase I and II enzyme functions to include now a broader range of tissue responses essential for parsing human liver toxicity.

Incentive to create new *in vitro* models arises from the massive cost of candidate drug failures due to liver toxicity [2], the low correlation between animal and human liver toxicities [3] and increasing challenges faced by the use of existing whole animal studies including animal welfare, legal/ethical issues, high costs, time, limited throughput, dose extrapolation issues and the other limitations of existing *in vitro* systems [4–7].

In vitro liver systems that are currently used in pharmaceutical drug discovery and development laboratories for evaluating drug metabolism, clearance and toxicity include freshly isolated primary hepatocytes, plated hepatocytes and immortalized cell lines, microsomal preparations, precision-cut liver slices, isolated perfused livers and complex organotypic reconstructed tissue [8–10]. Cryopreserved primary human hepatocytes remain the preferred method for studying *in vitro* drug metabolism and hepatic drug uptake. The activity of Phase I and II enzymes in these cellular systems generally remains stable enough over time scales required to gain both quantitative and qualitative information on metabolic conversion (typically on the order of several hours) [11–15], although analysis of enzyme induction, which requires more culture time, can be impaired [9]. Additionally, cyropreserved hepatocytes in suspension are a model system for assessment of hepatic drug uptake mediated by sinusoidal transporters responsible for influx of xenobiotics into the liver [16,17]. When the cells are plated in static cultures, they can be used to capture a significant fraction (~ 50%) of known toxicity events in small molecule drugs [18]. Thus, such culture systems retain an important place in drug development.

There remain many areas in which improved test systems can provide further value, motivating development of more organotypic culture schemes. For example, the utility of hepatocyte cultures to quantify the toxicity of molecular therapeutics such as gene therapy agents is limited due to the complex interplay between the hepatocytes and non parenchymal cells, especially immune cells [19].

Often, a significant challenge for studying and understanding mechanisms of toxicity is the need to administer several doses in vivo before observing a toxicological outcome, as exemplified by the drug troglitizone [20]. Because acute dosing does not often identify the true toxic potential of a molecule, more sophisticated secondary *in vitro* screens for assessing drug toxicity that maintain liver function over a time course that permits long-term multiple dosing of a new drugs are desirable.

Toxicity mechanisms for molecular therapeutics are also often complex [21,22]. Drugs that become toxic following metabolism may seem benign in culture systems in which the activity of CYP450s and other drug metabolizing enzymes are greatly reduced. Drug toxicity can arise from a wide range of mechanisms beyond those that can be discerned in acute dosing assays; hence, a second tier of analysis is needed to further probe candidate compounds that operate by mechanisms such as those involving immune or other cell interactions that manifest themselves over longer-term exposure to drug [2,23–27]. In an attempt to retain liver phenotype and function, various approaches have been used with the common aim of preserving or creating an in vivo-like environment. In this paper, we cover some of the approaches and design considerations in building a liver tissue model and describe at length a 3D liver model built in our laboratory that has been applied in the study of drug clearance and safety.

2. Evolving trends in liver tissue engineering in vitro

Hepatocyte metabolism plays such a central role in mediating liver responses to drugs that most liver tissue engineering efforts focus on preservation or enhancement of hepatocyte function, with other cell types often viewed as playing supporting roles. However, hepatocytes in vivo are complex metabolic cells whose function is dependant on their microenvironment, which not only consists of direct cell–cell and cell–matrix interactions but also myriad diffusible factors secreted by nearby non-parenchymal cells (NPCs) [28] as well as chemical gradients caused by the unique hepatic circulatory pattern [1,29,30]. The complex interplay of signals within and along the sinusoid result in gradients of hepatocellular function along the sinusoid in both homeostasis and pathological response [29,31,32]. Approaches to preserve hepatocyte function in culture have ranged from simple interventions such as adding medium components and growth factors resembling serum or changing the substrate to provide signaling akin to extracellular matrix to more complex attempts to preserve or create 3D cell–cell architecture (liver slices, spheroids etc.) or even perfusing the systems to recapitulate the cues provided by the flow of blood [33–41]. Described below are three general trends in the field of liver tissue engineering.

2.1 Towards heterotypic co-cultures

Historically, in vitro studies for drug metabolism and toxicity have used purified hepatocyte populations. While these systems have shown great utility in the drug discovery process (and will continue to play an important role), there are many areas in which enhanced models can add tremendous value. For example, it is well documented that NPCs in the liver, including Kupffer cells and endothelial cells, can contribute directly or indirectly to drug induced hepatotoxicity [42–48]. Identifying these toxicities *in vitro* will require a further level of testing using systems that contain more than hepatocytes alone. Indeed, short-term hepatocyte co-cultures with Kupffer cells have demonstrated synergistic interactions between xenobiotics and lipopolysachcharide (LPS) [49].

Another major driver for including NPC types is the enhancement of hepatocellular phenotype. An abundance of literature over the past few decades has shown the beneficial effects of each of the NPCs and other cell types in not only extending the survival of hepatocytes, but also in maintaining its differentiated state [50–61,63]. The positive effect is

attributed to the heterotypic direct cell–cell interactions as well as matrix and diffusible growth factors and cytokines secreted by the NPC types. For example, Kupffer cells release both proproliferative (e.g., TNF-α, IL-6) and anti-proliferative cytokines and signals (IL-1, TGF-β) [62–65]. TNF-α released from the Kupffer cells has been shown to downregulate the enzyme CYP2B1 in hepatocytes in co-cultures and might play an important role in the modulation of xenobiotic metabolism in liver [66]. Hepatocyte growth factor is a mitogen for hepatocytes and is synthesized by quiescent hepatic stellate cells [67] and co-culture with stellate cells improves hepatic function [68]. Studies using co-cultures of hepatocytes with sinusoidal endothelial cells [50,51] demonstrated that these cell types support long-term hepatocyte functional activity. The interaction has been shown to be reciprocal in enhancing the survival and retention of phenotype of the NPCs as well [69,70].

Just as cell density plays a prominent role in governing function of hepatocyte culture – cells must generally be plated at densities < 40,000 cells/cm^2 to show robust DNA synthesis with mitogen simulation and densities above ~ 50,000 cells/cm^2 to retain high levels of differentiated function [71,72] – the geometry of interactions between cells in co-cultures can influence outcome. Co-cultures initiated by stochastic seeding often evolve islands of two cell types; micropatterning approaches allow the degree of interactions between two cell types to be controlled more precisely on the culture substrate, with some improvement in function [61,63,73]. Whether 2D micropatterning approaches can capture all facets of physiology relevant for predicting toxicology remains an open question. Hence, development of 3D systems remains an attractive path forward for toxicology.

2.2 Towards 3D architecture

The idea of direct cell–cell and cell–matrix interactions promoting survival was suggested and subsequently demonstrated by reports that maintenance of differentiated phenotype in hepatocytes was enhanced by sandwiching mono-layers of cells between layers of extracellular matrix [37,38,74–78]. Studies of various tumor models also long since demonstrated that maintenance of differentiated phenotype was enhanced when cells form spheroids or multi-layer cell aggregates [79,80]. Formation of these structures requires cell–cell interactions mediated by E-cadherin, an intercellular adhesion molecule known to regulate hepatocyte cell functions [81,82]. The spatial constraints imposed by a 3D environment on cells determine how they perceive and interpret biochemical cues from the surrounding microenvironment [83–87]. Additionally, 3D architecture provides another dimension for external mechanical inputs and for cell adhesion, dramatically affecting integrin ligation, cell contraction and associated intracellular signaling [88,89]. In a 3D environment, the surrounding extracellular matrix both controls solute diffusion and binds many effector proteins, such as growth factors and enzymes, thereby establishing tissue-scale solute concentration gradients, as well as local pericellular gradients [1]. For liver, the earliest versions of fully 3D systems consisted of spheroids either of hepatocytes alone or co-cultured with NPCs [68,90–94]. These demonstrated a better retention of hepatocyte phenotype and function. Again, challenges in the stochastic nature of seeding cells in standard culture formats have driven micropatterning approaches to control size and geometric interactions between spheroids [95].

Another early 3D system was the liver slice. Many of the initial hurdles with liver slices (i.e., reproducibility) have been addressed with newer precision slicing systems and advanced incubation methods [96,97]. When slices are too thick, there are diffusion limitations into the tissue at the center of the slice and when slices are too thin, there is too much necrotic tissue from the cut relative to normal tissue inside the slice. Currently, liver slices can survive and function for 24 – 72 h making them useful for some toxicology applications [98–100]. Liver slices are also widely used to study liver fibrosis [101–103].

Major challenges in 3D cultures include the relatively short penetration depth of oxygen and macromolecules in 3D liver culture (~ 0.1 mm), and provision of appropriate mechanical stimuli in the absence of perfusion through the local tissue microenvironment. These challenges have driven development of more complex 3D set ups across both applications areas of liver tissue culture, bioartificial liver devices and microscale laboratory/testing models [104–106].

2.3 Towards flow based systems

One of the major limitations when creating dense 3D tissue structures is the delivery of oxygen to the tissue [1]. Efforts at ensuring adequate oxygen delivery in 3D systems have driven the design of systems that incorporate fluid flow across or through the tissue. Most efforts in this regard arise from the needs of large-scale systems for maintenance of cell viability during extracorporeal liver support, and prominently include membrane-based reactors (in which cells are cultured outside semi-permeable, hollow-fiber membranes), perfusion reactors (in which cells are grown in porous scaffolds and fluid is pumped around them) and stirred suspension-culture reactors (in which aggregates of cells are kept in suspension) [40,41,107–112]. The large scale of these devices makes them impractical to use in basic research applications, although concepts from large-scale systems have been applied to scaled-down versions of these systems [113]. Perfusing culture medium thorough the tissue can overcome oxygen transport limitation by increasing the amount of oxygen available to the tissue [114,115]. Perfusion flow can be achieved in small set ups with the help of microfluidic pumps and valves designed to circulate culture medium through small culture units on the order of a few thousand cells [116–119]. In addition to providing nutrients and oxygen, flow can be used to control the oxygen gradient across the tissue, a feature of particular significance in the liver on account of the heterogeneity in phenotype and metabolic patterns seen in vivo between periportal and pericentral hepatocytes in the hepatic lobule [29,30,120–123]. Additionally, flow of medium through the tissue can potentially recapitulate the mechanical cues provided by blood flow in the sinusoids [1].

The features and incremental benefits of these evolving models over traditional hepatocyte systems are listed in Table 1.

3. Considerations and design principles for perfused 3D liver toxicity models

There are many factors that play a role in creating a predictive model for drug hepatotoxicity. The first is that the test system must represent the population who will

ultimately receive the drug. Second, the exposure of the compound to the system must be of sufficient duration and concentration to generate a relevant response. Next, appropriate end points must be measured and either mimic or correlate to clinically observed end points indicative of toxicity. Various assays for toxicology have been discussed expansively in literature [18,124–127]. Finally, and probably most importantly, the data must be used appropriately to make decisions that will guide the development process. It is critical to understand the limitations of any test system and how they influence results. Another difficult challenge is extrapolation of concentrations and results from an in vitro model to in vivo, or between species. Understanding there are many elements that influence the predictive nature, we focus here on the test system. To design an in vitro model of liver that functions like in vivo liver with regard to drug metabolism, immune response and toxicity, the following factors need consideration.

3.1 Cell source, type and quality

Because significant species differences can exist in the metabolism and toxicity of various compounds [128,129], selection of cells to use in the system (be it from human or various animal species) should be done with the understanding of potential limitations in translation to the clinical setting. The most common sources are freshly isolated or cryopreserved enriched fractions of hepatocytes [8,9]. Addressing the need for a reproducible source of highly functional cells, an immortalized human cell line that can be induced to express a high degree of differentiated function has been commercialized [130]. Promising, but still only on the verge of commercial application, are the many approaches to differentiation of tissue-specific (liver) or pluripotent stem cells (including mesenchymal and embryonic) into mature hepatocytes [131–136]. Regardless of the type or species of cells, they need to be reliably sourced with minimal variability from batch to batch for the model to provide reproducible results. In the case of human cells, given the great inter-individual variability of cytochrome P450 patterns between donors, prediction of drug metabolic profiles, drug–drug interactions and toxicity of new drugs should be investigated in hepatocytes from several donors [6,8].

When maintaining heterotypic cultures, the relative numbers and dedifferentiated phenotype of different cell types can change during culture. It is thus important to ensure the functional phenotype of cells in the system is maintained throughout the assay duration. An array of markers can be used for functional activity; for instance, probe compounds can be used to check maintenance of specific drug metabolizing enzymes [8,137], albumin can be a functional marker for hepatocytes or the membrane antigen SE-1 can reflect functionality and differentiated phenotype of rat sinusoidal endothelial cells in co-cultures. Quantitative analysis of these markers is a continuing challenge, making it essential to standardize seeding numbers and operating conditions.

3.2 Determining length scales and flow rates based on predicted tissue requirements

As with the liver in vivo, in a 3D hepatic tissue unit, concentration gradients can exist for soluble and diffusible culture-medium components that are consumed or produced by cells, from oxygen to basic nutrients or secreted factors. Gradients of oxygen tension in vivo give rise to the phenomenon of zonation, driving differences in phenotype across the lobule. At

periportal regions where oxygen tension is higher and gluconeogenesis occurs, oxygen consumption is higher with respect to pericentral regions where oxygen tension is lower and glycolysis occurs. In addition to oxygen consumption rates, the distribution of drug metabolizing enzymes and transporters is heterogeneous in the liver. Most CYPs are located in the perivenous region [29,30]. Transporters, such as OATP1A4 are expressed more abundantly in the midzonal perivenous region and not the periportal region [138]. However, some transporters (e.g., MRP2 and MDR1) have been shown to be expressed equally in both periportal and perivenous regions of the liver [139,140]. In the liver, the oxygen tension is typically lower than other organs due to the portal blood supply. The baseline metabolism and functions occur typically in environments ranging from 30 to 90 mmHg O_2, a value significantly lower than atmospheric oxygen tension (160 mmHg O_2) [141].

In vitro, oxygen is rapidly depleted due to its relatively low solubility in culture medium and the high metabolic activity of the cells. To achieve a physiologically relevant 3D microenvironment, it is important to design length scales based on known or predicted cellular requirements. Experimental values of cellular utilization are available in literature [142,143] and can be used in conjunction with diffusion and flow parameters as the basis for determining the upper bounds for length scales. In cases of floating spheroids where all the surfaces are exposed to oxygen saturated media, oxygen transport by diffusion can support a cellular mass of greater diameter than when oxygen transport only occurs across one plane, such as tissue packed into a well. For example, based on reported oxygen consumption rates [144], a spherical mass of tissue at an elevated oxygen consumption rate would need to be smaller than 380 μm to allow oxygen to diffuse into the center. Likewise, a static culture would need to be < 110 μm deep to allow for enough oxygen to reach the bottom layer of cells. The depth of static medium above a tissue surface also imposes diffusion limitations. At cell densities regularly used in tissue culture, a depth of 2 mm can reduce oxygen availability at the cells to 10 – 50% of the oxygen concentration at the air–liquid interface [145].

To overcome diffusion limitations, flow can be used to transport oxygen to the tissue. In a system that is perfused, or when culture medium flows across the tissue surface, it is important to consider the effects of shear stresses. Higher than physiological shear rates, such as those seen after partial hepatectomy, are known to perturb the different cell types, giving rise to a range of cell signaling events and cell growth seen in hepatic regeneration [146]. It has been shown that while a small amount of shear stress is beneficial to cultures of hepatocytes (below 5 dynes/cm^2), larger amounts of shear are shown to be damaging [147–149].

3.3 Scaffold material and geometry

A scaffold is used to provide support for 3D organization of cells. The composition, architecture and mechanical properties of the scaffold work together to dictate tissue morphogenesis and continued evolution of tissue function. A facile means to create scaffolds is by drilling cell-holding features into a thin polymer sheet of materials ranging from polystyrene to polycarbonate, and post-coat the scaffold with extracellular matrix [150]. Finally, it is critical that a scaffold allows access to tissue for measurement of the desired

end points. Depending on the application, scaffolding should be conducive to imaging or harvesting the cells for protein or gene expression levels. Although relatively stiff materials such as polystyrene provide mechanical stability to support cells under flow, the growing appreciation that stiff substrates activate stellate cells [151] and influence hepatocellular function [152] is driving development of gel-based scaffolds that provide a closer match to liver extracellular matrix.

4. A 3D perfused liver bioreactor

A liver tissue-engineered perfused bioreactor that shows significant promise for studying drug toxicity and metabolism has been described recently [153,154]. The core of the device is a scaffold (Figure 1A) containing a matrix of 3D liver tissue units that mimic the liver capillary bed. Culture medium continually circulates through the scaffold and bioreactor (Figure 1B). To enhance throughput as well as integration into existing automated cell culture paradigms, the device was designed to mimic the format of a multi-well plate, and houses 12 isolated bioreactors each with an integrated micro pump for controlling flow (Figure 1C). The capillary bed is formed by seeding a suspension of liver cells into a reactor well; the cells adhere to the scaffold contained in the well and form tissue-like structures under continuous perfusion. As culture medium flows through the tissue bed, oxygen is consumed resulting in a gradient across the tissue that is similar to the oxygen gradient in the in vivo liver sinusoid [154]. The system was designed such that this gradient and the actual concentrations can be tuned based on flow rate and other operating parameters [154]. As a result, mechanical parameters in the system can be easily adjusted and coupled with functional output to help optimize the system effectively. Several generations of these 3D perfused bioreactors have been developed and each generation maintains a core scaffold that is continually perfused with culture medium [33,34,117,118]. In the most recent version, several reactor chambers were integrated with their associated pumping systems onto a single plate for increased throughput, reliability and ease of use [154].

This perfused liver bioreactor system has been well characterized in the literature and has been shown to enhance the maintenance of liver function in many respects [34,117–119]. Non parenchymal cells when co-cultured with hepatocytes in this system have been shown to retain expression of SE-1, a marker of sinusoidal endothelial cell phenotype for as long as 14 days [119], a period much longer than otherwise reported in literature [155,156]. Kupffer cell presence, as evidenced by staining of the functional marker ED-2, has also been demonstrated after 2 weeks of culture (Dash, Griffith and Tannenbaum, manuscript in progress). Here, we describe how the liver environment is maintained, and how this environment enhances the maintenance of liver function, and finally provide an example of the system’s utility.

4.1 Maintenance of liver function

Expression and function of P450 enzymes is rapidly lost in static culture preparations [157–159] whereas it seems to be well maintained in rat hepatocytes cultured in the 3D perfused environment (Figure 2) [117]. In addition to gene expression, the function of selected P450 enzymes has been assessed. Assays were performed by culturing human and rat hepatocytes in the 3D perfused environment for 4 – 6 days, followed by incubating a probe compound in

the system for 24 h. The disappearance of probe compounds was measured using LC/MS/MS, and clearance was calculated from these values. Metabolic activity in the perfused bioreactor was compared with historical data from a standardized 4-h suspension assay of freshly thawed rat or human cells (Table 2) [160].

One application for systems that maintain metabolic function is the prediction of in vivo clearance rates. Figure 3 shows the alignment of human in vivo data with in vitro clearance in the perfused 3D liver for a set of nine compounds (Sevidal, Kellet and Obach, manuscript in preparation). Assays were performed using cultures of cryopreserved human hepatocytes, lot Hu4000 (CellzDirect, Inc. Durham, NC, USA). Following 6 days in culture, the probe compound was incubated in the system for 3 days and medium samples were taken periodically. Clearance values were calculated based on disappearance of the parent compound throughout the incubation period. Good alignment between in vitro and in vivo was observed (Figure 3). The exception in this study is the compound meloxicam, in which the perfused microenvironment under-predicts in vivo clearance. This was potentially the result of genetic variation in the specific donor tissue used for the study, as cells from the same donor also under-predict clearance of meloxicam in a 4-h suspension assay (data not shown).

5. Application of the 3D perfused bioreactor as a model for drug toxicity

Drug induced liver injury (DILI) is the number one cause of death from acute liver failure [161] and the most common cause of drug withdrawal from the market [162]. Broadly, DILI can be categorized into DILI type I (e.g., predictable from animal models, high frequency of occurrence, dose-dependent) and DILI type II (rare occurrence, difficult to predict from preclinical species, little correlation with dose). DILI type II, also referred to as idiosyncratic hepatotoxicity, is a major concern in drug development due to its unpredictable nature and appearance at ordinarily non-toxic doses. One hypothesis for the mechanism responsible for these idiosyncratic responses is due to the presence of inflammatory response elements (e.g., cytokines, chemokines). Numerous in vivo studies have shown that treatment with certain drugs, such as ranitidine, trovafloxacin and diclofenac, in a modest inflammatory setting triggered by bacterial LPS can precipitate idiosyncratic liver injury in rats [23,42,163]. Corresponding in vitro experiments required conditioned medium from neutrophils to elicit toxicity, emphasizing the necessary role of other cell types in the inflammatory response [23]. Other studies that used Kupffer cells and hepatocytes to create an inflammatory response highlight the need of direct cell–cell contact between different cell types *in vitro* to achieve this effect, and point to a reciprocal interaction between the two cell types [43].

To design a liver model for drug toxicity capable of maintaining an inflammatory component, we used the micro perfused reactor system described in section 4. Monocultures containing an enriched population of hepatocytes (≥ 95%) and co-cultures containing NPCs in near physiological proportions (Table 3) were seeded into 12 wells in the reactors. After 4 days, six of the wells were stimulated with 1 μg/ml LPS. Elevated levels of both pro-inflammatory (IL-1, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines as well as higher levels of nitrites/nitrates observed in the medium after 48 h demonstrated that the co-cultures

containing Kupffer cells exhibited a robust inflammatory response to the LPS compared to the hepatocyte only cultures (Figure 4).

Under these inflammatory conditions, different test drugs known to exhibit idiosyncratic toxicity were then tested using this model along with non-idiosyncratic controls. Cultures were set up as before and the test compounds were added in non-toxic doses to LPS stimulated and DMSO treated control wells. Medium was sampled over 48 h for markers of toxicity and the tissue was stained for cytotoxic response. Over the dose range studied, treatment with the compounds or LPS in isolation caused minimal toxicity in the co-cultures (as evidenced by lactate dehydrogenase release). However, when LPS was administered in combination with known idiosyncratic toxicants (e.g., ranitidine), a marked toxic response was observed (Figure 5) as compared to control drugs from the same therapeutic group (famotidine). Currently, a wider range of idiosyncratic drugs paired with their non-toxic counterparts are under evaluation.

6. Choosing an appropriate toxicity model

Each of the different approaches listed in Table 1 (or a combination of them) incorporates novel enhancements to current cell culture approaches in an attempt to more closely mimic in vivo liver physiology and function. These features introduce a level of complexity to account for several factors that have a role in influencing cellular phenotype, function and behavior as well as response to a drug or xenobiotic. The key advantages of such complex heterotypic systems is that they clearly result in better maintenance of basic cellular function [55,117,164–168] and that they begin to capture the function of entire organs rather than isolated cells. By virtue of being more like the in vivo setting, these culture systems approximate drug disposition and toxicity in intact preclinical and clinical species in terms of physiological relevance as compared to isolated primary cells plated on a culture dish in a static environment. The applications of these complex models can be specifically tailored to individual needs by manipulating the cell types (as described above) and operating conditions; for instance, varying shear stress or nutrition/oxygen gradients. The increased longevity of viable functional tissue allows for experimental designs such as repeat dosing and long-term evaluation that cannot be achieved in conventional cell cultures with primary cells. Having a system amenable to imaging can provide visualization of cellular level events and morphological changes in a 3D tissue context. Last but not the least, the incorporation of human cells into these systems provides a platform to test drugs in a clinically relevant setting, thereby, bridging the gap between animal and human studies.

While creating a heterotypic model, we have tried to incorporate most of the cell types present in the liver. Kupffer cells, which form almost 80% of the resident macrophage population, when stimulated with LPS in our system elicited an inflammatory background to test the drugs. However, one has to recognize that responses arising due to the recruitment of circulating immune cells such as T lymphocytes and neutrophils are responsible for further mechanisms of toxicity seen in vivo, and these may need to be captured in a different model designed accordingly.

There are also challenges that come with increased complexity that make simpler models preferable in some cases. Cost and capacity are important considerations when selecting an appropriate model at a given stage of drug discovery and development and more simplistic models that address specific research questions will continue to play a crucial role in drug discovery. Rather than replace these models, tissue engineered systems will probably be used in parallel or at later stages to augment data generated in more simplistic systems. Nonetheless, the ultimate choice of model should be driven by the scientific question posed and what physiologic and mechanical components are critical to generate the experimental outcome in the in vitro setting. Given the importance of drug induced liver toxicity in the ultimate development and success of any drug, we recommend having several models available to methodically evaluate compounds at several stages of drug discovery and development. Ultimately, a model that incorporates flow, NPCs and hepatocytes, as well as the ability to evoke an inflammatory response to endotoxins and other immune activators, may be the appropriate tool for understanding mechanisms of toxicity and drug disposition. This model likewise would better enable preclinical study design (including species selection), and better predict the possibility of adverse effects in clinical trials for novel therapeutic agents.

7. Conclusion

Technological strides in the field of liver tissue engineering provide opportunities to transform standard cell culture methods into more complex systems that retain liver function over time. As a direct result, more predictive and reliable tools for understanding drug toxicity and disposition are now emerging. We have discussed some of the evolving approaches, and how they can be combined to create a 3D heterotypic co-culture system that fosters survival of functional hepatocytes and NPCs. Studies in the bioreactor system described above demonstrated its ability to retain gene expression levels for several cytochrome P450s closer to freshly isolated levels as compared to static cultures as well as to maintain metabolic function allowing for prediction of clearance rates similar to in vivo values. Benefits of using this liver bioreactor include the ability to study a metabolically competent system over a long period of time. This provides an important advantage for assessment of the metabolism of low clearance compounds, the formation of secondary/tertiary metabolites and the impact of multiple dosing on drug disposition. It is also crucial for studying the safety of compounds when toxicity is potentially mediated by metabolites. Finally, the ability to compare results between human and animal species is very useful for designing and understanding preclinical pharmacokinetics and safety studies.

To explore this system as a possible model for predicting idiosyncratic toxicity, we utilized the presence of NPC–hepatocyte interaction and characterized a reproducible response to LPS as an inflammatory stimulus. The resulting model was used to demonstrate sensitivity of certain drugs such as ranitidine at non-toxic doses in an inflammatory environment and could serve as a potential model for idiosyncratic hepatotoxicity.

8. Expert opinion

A decade ago, the utility of hepatocyte cultures in preclinical drug development was just becoming appreciated as standard methods for isolation, shipment and culture of human hepatocytes made it possible to develop *in vitro* tests that correlated with in vivo toxic events for a subset of compounds known to be toxic. Multi-well plate cultures of hepatocytes have passed an important milestone in that they can be utilized to capture known toxicities with minimal false positive outcomes (i.e., reporting a known safe compound as toxic), although a large fraction of known toxic compounds fail to be identified using these technologies [18,169]. Multi-well plate-type hepatocyte cultures thus have an important role in early screens, but must be augmented by further approaches to improve predictive power. In response to this success with its attendant shortcomings, the field is moving in several directions simultaneously. At one end of the range, technologies are emerging that push versions of accepted assays for hepatocellular metabolism and toxicity into cheaper and more robust high-throughput modes through miniaturization [170]. Miniaturized cell culture formats are moving into the commercial realm at a rapid pace for both general culture and specific applications; hence, success of these formats for toxicology is highly probable. Still, while such technologies have the potential to greatly increase the number of assays and the quantitative information available for each compound and to potentially include some non-liver target cell effects [171], a fundamental question remains whether they will offer a significant advance in predictive power (i.e., whether they will capture a significantly greater fraction of toxic compounds).

Several emerging efforts suggest that standard hepatocyte cultures, and their miniaturized counterparts, will be pushed toward better performance (i.e., greater statistical correlation with known toxic compounds) by judicious combinations of culture conditions, measurement methods and data interpretation that incorporate mechanistic hypotheses. For example, multimode imaging can reveal more subtle effects on cells [18] and combinations of inflammatory cytokines with drugs capture toxicities that are not evident without the co-morbid stress [169]. These approaches, combined with systems biology [172], will improve the predictive power and lower cost in the next 5 – 7 years. The future of these technologies depends on whether they can be validated to improve prediction of toxicants; that is, whether they can capture not only toxicities identified in existing preclinical screens, but also a significant number of toxicities that were missed with current screens, but were identified in clinical trials. If proven through such validation, these tools would establish a platform for better prediction of clinically adverse events and could decrease the post-marketing failures seen with several recent therapeutic agents. We speculate that these technologies may become integral aspects of regulatory submissions to help support safety and disposition information on novel therapeutic agents.

But although we foresee dramatic improvement in these hepatocellular assays, we anticipate that tissue engineering approaches will provide essential further screens for many compounds, and possibly a primary screen for new classes of drugs. As one simple example, we have highlighted the hypothesis that a substantial percent of idiosyncratic toxicities arise from co-morbidity of drug plus inflammation. A preliminary study in hepatocytes treated with inflammatory cytokines shows that predictive power improves, but still falls far short

of desirable [169]. Although resolution in this assay might be improved by manipulating protocol variables such as timing of cytokine additions, fed/fasted state of cells and hypoxia, our view is that the complex interactions that lead to toxicity will be difficult to replicate economically in a 2D screen compared to a 3D screen, as many of the variables that must be represented individually in different wells in 2D screens (hypoxia, cytokine combinations) are captured naturally in a single well of a 3D culture due to the presence of nonparenchymal cells and zonation along the path of flow.

We anticipate that the need for tissue-engineered models will be underscored by the needs for preclinical assessment of molecular therapeutics such as siRNA and other gene therapy vectors, which are often taken up by liver even when they are not intended to be. Molecular therapeutics have a highly complex interaction with liver, which in addition to metabolism is the largest immune organ in the body. Although the focus is typically on the Kupffer cell–hepatocyte interactions when clearance and toxicity of molecular therapeutics are discussed, the prominence of sinusoidal endothelium as players in the immune response of liver is rapidly emerging [173]. Sinusoidal endothelial cells are very difficult to culture in a differentiated, functional state [119]; hence, co-cultures that replicate features of the complex interactions among cells in the liver sinusoid will be needed to understand clearance and toxicity of these rapidly-emerging new therapeutics. Finally, tissue engineering will almost surely be needed to assess chronic responses, including those involving trafficking of bone-marrow-derived cells into liver [174].

What is the best path for development? Advances are occurring rapidly in several fields that together impinge on creation of robust systems for tissue engineering. Synthetic biomaterials that are easier to process and store, and have more reproducible lot-to-lot properties, are moving into the mainstream in many areas of cell biology [175]. These are being combined with advances in microfluidics to create integrated devices that can be translated into pharmaceuticals labs [34,115,117,119,154,176,177]. As these tools come on line, their ultimate success in transforming predictive toxicology will rely on using them to address well-posed mechanistic questions and on their integration with systems biology approaches that can parse several types and hierarchies of information.

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Declaration of interest

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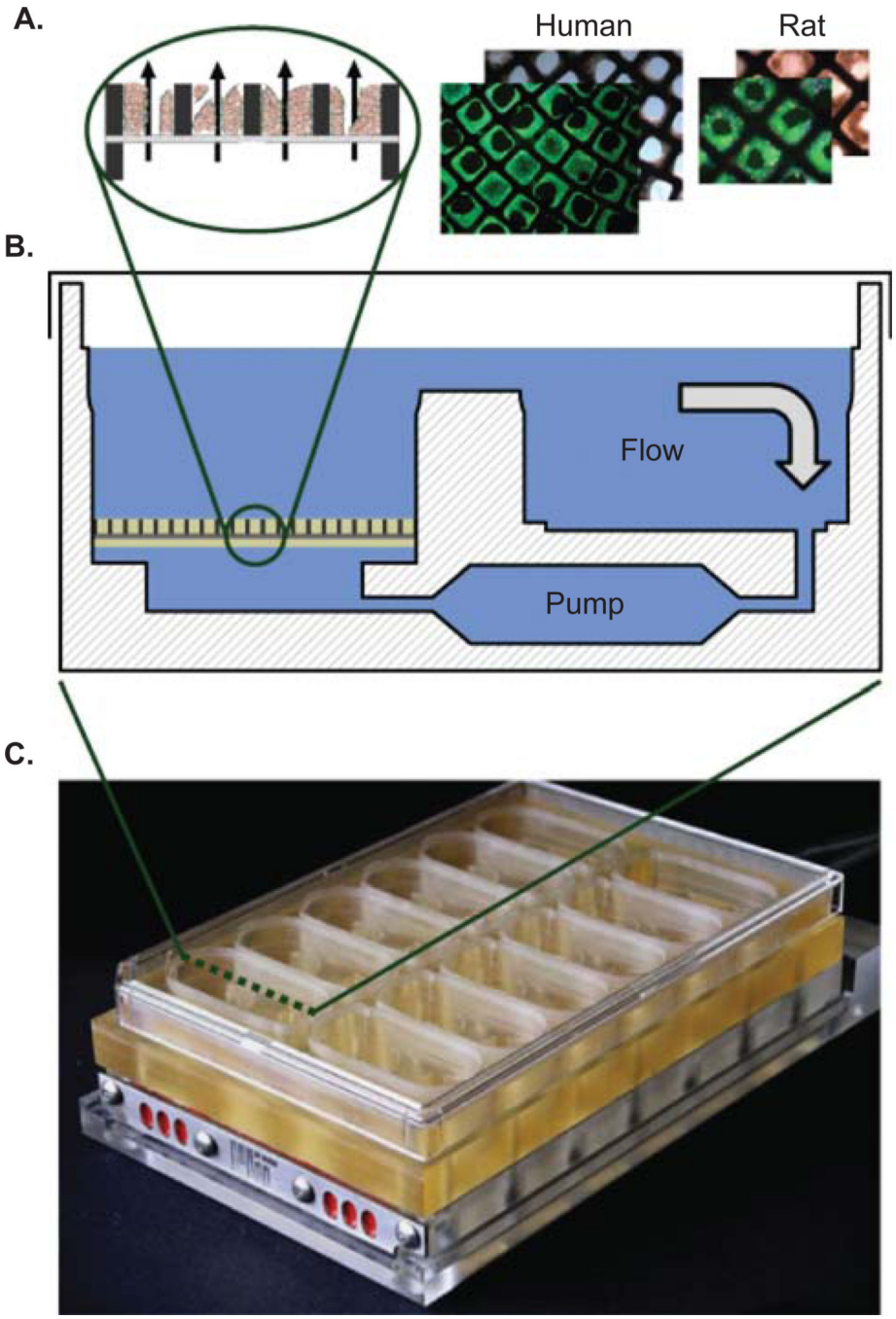


Figure 1. A live-dead stain of human and rat hepatocytes cultured in the scaffold alongside a schematic cross-section

(**A**). The scaffold resides within a well of the bioreactor (**B**) and there are 12 isolated 3D perfused bioreactors on each culture plate (**C**).

Photograph courtesy of Karel Domansky, MIT 2008.

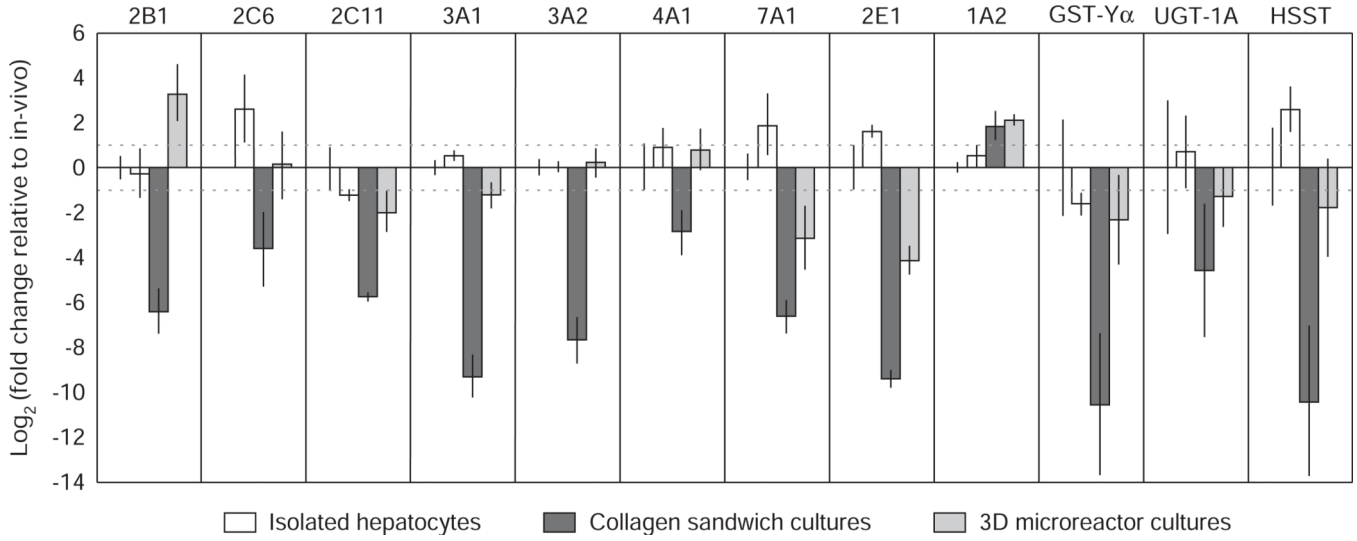
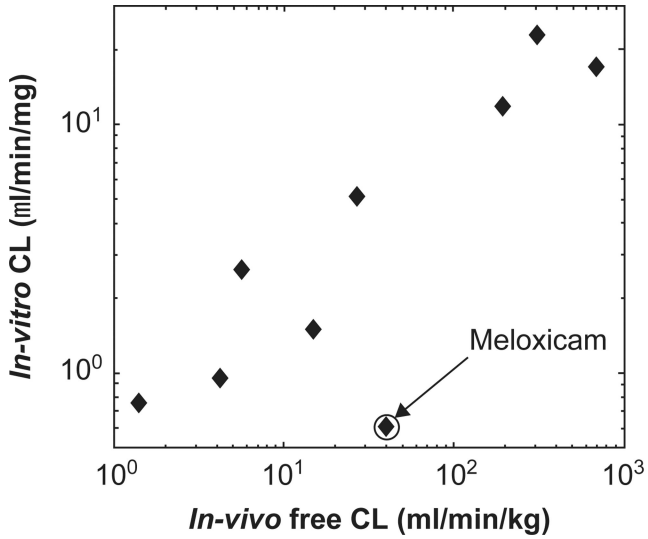


Figure 2. RT-PCR analysis of relative expression of Phase I and II drug metabolizing genes

Constitutive expression of Phase I and II genes in isolated rat hepatocytes, collagen sandwich culture (day 7) and 3D microreactor (day 7) cultures expressed as log twofold change relative to rat liver *in vivo*.

Reproduced from Sivaraman *et al*. 2005 [117] with permission from Bentham Science Publishers Ltd (2009).

RT-PCR: PCR after reverse transcription of RNA.



Drug	Enzyme	CL	Class
Tolbutamide	2C9	Low	Acid
Meloxicam	2C9 & 3A4	Mid	Acid
Diclofenac	2C9	High	Acid
Disopyramide	3A4	Low	Base
Metoprolol	2D6	Mid	Base
Verapamil	3A	High	Base
Theophylline	1A2	Low	Neutral
Methylprednisolone	3A	Mid	Neutral
Midazolam	3A	High	Neutral

Figure 3. Measured clearance in 3D perfused culture of cryopreserved human hepatocytes compared with in vivo human CL for nine compounds: Sevidal, Kelly and Obach, manuscript in progress

CL: Clearance.

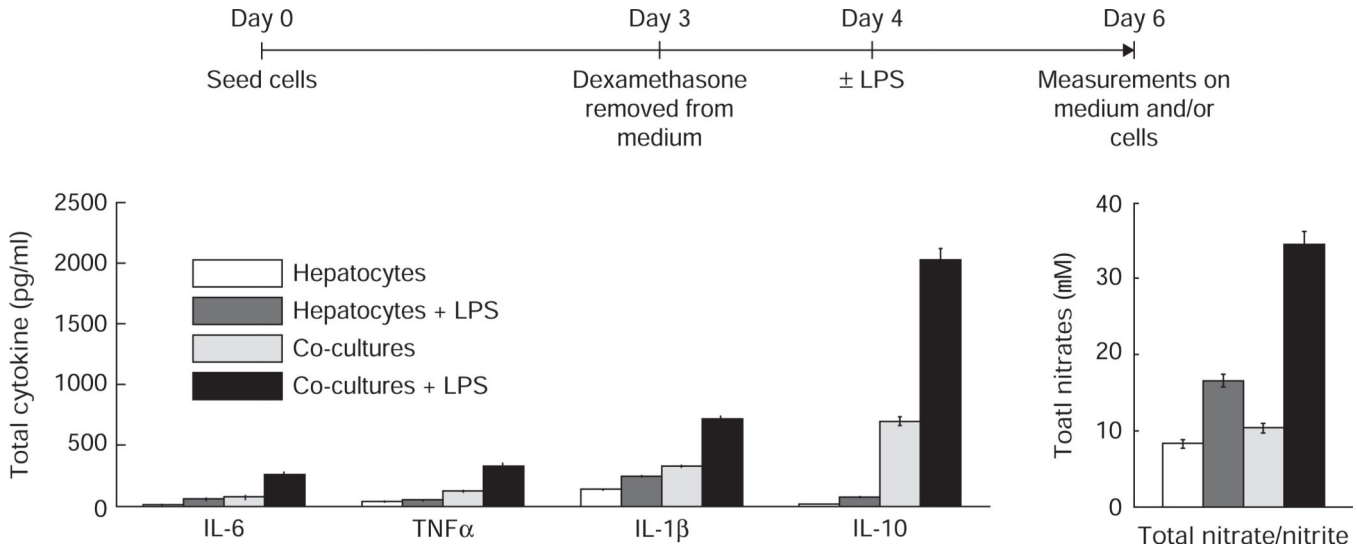


Figure 4. Monocultures or co-cultures (initial numbers shown in Table 3) were seeded into the reactors

After allowing the cultures to stabilize for 3 days, dexamethasone was removed from the medium and 24 h later, 50% the wells were stimulated with 1 μg/ml LPS. Measurements of cytokines and total nitrates/nitrites were made from the medium 48 h after treatment. The results show an enhanced inflammatory response in co-cultures. Dash, Griffith and Tannenbaum, publication in progress.

LPS: Lipopolysachcharide.

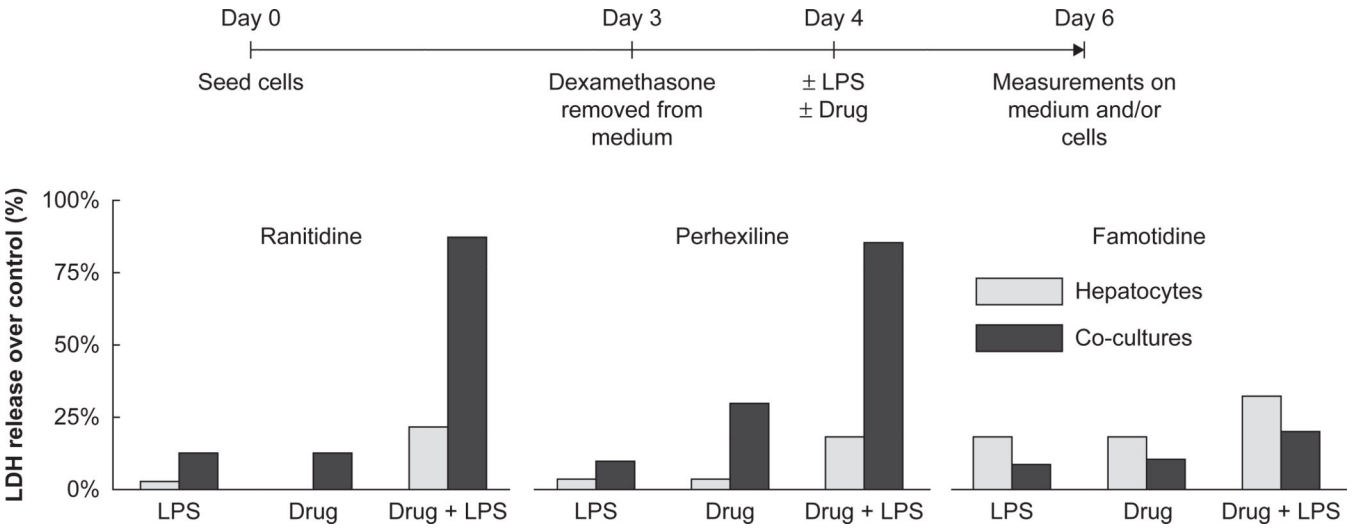


Figure 5. Monocultures containing a mostly hepatocytes (95%) or co-cultures containing NPCs in defined proportions were seeded into the reactors

After allowing the cultures to stabilize for 3 days, dexamethasone was removed from the medium and 24 h later, 50% the wells were stimulated with 1 μg/ml LPS and/or the test drugs (ranitidine (800 mg/ml), perhexiline (5 mmol), famotidine (175 mg/ml) or DMSO controls). Measurements of LDH release were made from the medium 48 h after treatment. LPS markedly increased toxicity of the known idiosyncratic drug ranitidine but not its corresponding negative control famotidine. Dash, Griffith and Tannenbaum, publication in progress.

LDH: Dehydrogenase: LPS: Lipopolysachcharide: NPC: Non-parenchymal cell.

Table 1

Evolution of in vitro liver tissue models

Approach	Specific feature	Observed benefit	Ref.
Co-culturing hepatocytes with other cell types	Hepatocytes with endothelial cells	Maintenance of differentiation and function	[50,51]
	Hepatocytes with stellate cells	Preserve hepatocyte function, hepatocyte proliferation	[52–54]
	Hepatocytes with Kupffer cells	Optimize hepatocyte function	[60]
	Micropatterned hepatocytes with NPCs	Preserve hepatocyte phenotype	[55,59]
	Hepatocytes with other cell lines	Preservation of hepatocyte function and phenotype	[56,58]
3D co-cultures	Hepatocyte-stellate cell spheroids	Preservation of hepatocyte function	[68,94,164,165]
	Layered hepatocytes and endothelial cells	Upregulation of metabolism genes	[167]
	Hepatocytes with cell lines	Preservation of hepatocyte function	[166]
Flow based systems	Continuous flow around hepatocytes in spinner baskets	Higher levels of DNA synthesis	[168]
	Perfused scaffolds with 3D hepatocyte aggregates	Better morphology and viability	[118]
	Perfused scaffolds with 3D hepatocyte-NPC aggregates	Sinusoidal phenotype maintained long-term	[119]

NPC: Non-parenchymal cell.

Table 2

Metabolic clearance of three compounds in rat and human

Suspension are taken from Pfizer Research Technology Center values, LiverChip values are day 6 for rat (two experiments) and day 4 for human (four experiments).

Drug	Enzyme	Clearance values (μL per min, per million cells)			
		Rat suspension	Rat LiverChip	Human suspension	Human LiverChip
Midazolam	3A4	35	38	13	13
Naloxone	Phase II	51	39	30	29
Propranolol	1A2, 2D6	61	48	19	8

Expert Opin Drug Metab Toxicol. Author manuscript; available in PMC 2014 July 25.

Table 3

Characterization of cellular input into bioreactors: hepatocytes were isolated by a modified two-step collagenase perfusion process described earlier [117] and isolates with viability > 90% were consistently used

NPC fractions were purified from supernatants based on published methods [119] and stained with different immunostains (SE-1 for sinusoidal endothelial cells, ED-2 for Kupffer cells, GFAP for stellate cells) before quantifying by flow cytometry. NPC isolates were consistently shown to have a viability of > 95%. Based on the proportions, defined numbers of each cell type were added back into reactors at the time of seeding.

Cell type	Function	Reference in vivo values	Seeding numbers/reactor well	
			Monocultures	Co-cultures
Hepatocytes	Main metabolic cells	60%	800,000 (95%)	400,000 (40%)
Endothelial cells	Filtration, secrete cytokines	20%	(1 –2%)	400,000 (40%)
Kupffer cells	Macrophages, inflammatory response	15%	(1 –2%)	150,000 (15%)
Stellate cells	Fibroblasts, store fat, secrete ECM	5%	(< 1 %)	50,000 (5%)

NPC: Non-parenchymal cell.