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Aggregate culture: A more accurate predictor of microcystin toxicity for risk assessment

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ABSTRACT

Aggregate or spheroid culture has emerged as a more biologically relevant method for screening pharmaceutical compounds and understanding exact mechanism of action. Here in, the aggregate approach applied to the freshwater toxins, microcystins, further unearths exact mechanism(s) of toxicity and provides a markedly improved in vitro predictor of toxicity. Microcystins result in acute intoxication by binding covalently to protein phosphatase 1/2A, resulting in hepatocellular necrosis, hemorrhaging and death. Hepatocellular uptake by organic anion transporting polypeptides (OATPs), in addition to other intracellular sequelae, is considered essential for toxicity. In aggregate HepG2, expression of OAT1B1 and OATP1B3 significantly increased relative to monolayer culture. Uptake of two fluorescently labeled substrates significantly increased in aggregates compared with monolayer, confirmed by inhibition of uptake with known competitive substrates. Increased reaction oxygen species (ROS) production occurred following a three-hour exposure of microcystin LR at concentrations from 100 nM to 100 μM, with reversal by ROS scavengers, in contrast with no response in monolayers. These results suggest monolayer culture inadequately predict intracellular effects of microcystins and support evidence that aggregate culture more closely approximates in vivo form and function. The approach results in more reliable prediction of microcystin toxicity in vitro.

1. Introduction

The acutely hepatotoxic monocyclic heptapeptide cyanotoxins, microcystins (MCs), produced worldwide by cyanobacteria, represent rigorously studied, but poorly understood toxins for which a more biologically relevant in vitro approach is needed. The identified MC congeners number well over 100 (Meriluoto and Spoof, 2008). The World Health Organization (WHO) provides a provisional drinking water guideline of 1.0 μg/l based on limited available data and risk assessment with respect to one congener, microcystin-LR. Major gaps relative to risk assessment for total MCs and exact mechanism of action remain (Dietrich and Hoeger, 2005) and must be addressed with a reproducible bioassay for toxicity to better protect public health (Monks and Moscow, 2010). Through inhibition of ubiquitously expressed protein phosphatase 1 and 2A (Runnegar et al., 1995), MCs impact numerous animal species (Campos and Vasconcelos, 2010; Dawson, 1998). Following ingestion, the toxin primarily targets the liver, resulting in cytoskeletal disruption (Hooser et al., 1991) and acute hepatic hemorrhage and necrosis (Hooser et al., 1989). In addition to the downstream effects of cytoskeletal disintegration through disruption of protein dephosphorylation, MCs bind with cellular thiols, deplete glutathione, result in mitochondrial dysfunction, alter patterns of DNA repair and cellular proliferation and generate reactive oxygen species (ROS) in cells other than

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in the liver (Campos and Vasconcelos, 2010; Gehringer, 2004).

The amphiphatic 900 to 1100 Da, generally hydrophilic MCs do not cross cell membranes easily and rely on the transporters OATP1B1 and OATP1B3, located along the basolateral sinusoidal membranes, for hepatocellular uptake (Fischer et al., 2010, 2005; Komatsu et al., 2007; Lu et al., 2008; Monks et al., 2007). Despite nanomolar sensitivities in transfected cell lines (Fischer et al., 2010, 2005; Komatsu et al., 2007; Monks et al., 2007) and potent cytotoxicity in primary cells and in vivo, monolayer immortalized cultures, including HepG2, remain viable at high μM concentrations 48, 72 and 96 h after exposure (Chong et al., 2000). Facilitated endocytosis techniques have also been employed (Jasionek et al., 2010), yet these models may exaggerate toxic effect relative to in vivo effects (Monks and Moscow, 2010).

Microscale approaches to aggregate or spheroid culture of primary cells and immortalized cell lines provide a relatively inexpensive technique for evaluating cytotoxicity that more closely approximates in vivo function (Khademhosseini, 2007; Khademhosseini et al., 2006). To date, little literature has focused on liver OATPs despite their importance in xenobiotic absorption, distribution and elimination. Primary rat liver cell aggregates maintained expression of basolateral and apical transporters, including the murine analog OATP1B2 (Sidler Pfandler et al., 2004), and multicellular spheroid formations of HepG2 cells using peptide nanofiber hydrogels demonstrated increased structural and functional polarity (Malinen et al., 2012).

While expression of OATP1B1 and OATP1B3 have been shown to be 1.5–2 fold higher in fresh human liver as compared with monolayers of HepG2 (Hilgendorf et al., 2007), aggregate HepG2 cells demonstrate improved expression and functionality, including distinct cortical actin organization in spheroids concurrent with up regulation of metabolic and synthetic genes and increased cytochrome P450 activity and albumin production as compared with monolayers of the same cell type (Chang and Hughes-Fulford, 2009). Cytochrome P450 expression and inducible expression in HepG2 aggregates substantially increases (Nakamura et al., 2011), along with immune and cytokine response (Liu et al., 2011). HepG2 aggregates may prove less susceptible to pharmacological agents as compared with monolayer culture (Li et al., 2008) because of increased expression of export pumps, MDR-1 and MRP-2 (Mueller et al., 2011; Oshikata et al., 2011).

To date, toxicity of MCs has only been evaluated in monolayer culture, likely underestimating uptake and extent of toxic effects. In addition, the mechanism of effect may be altered at various delivered concentrations, thus reducing understanding of mechanism of action, particularly across congeners. Given the increasing evidence in support of the in vivo like function of aggregates, we evaluated comparative expression of relevant transporters and comparative uptake and toxicity of MCs in monolayers and aggregate HepG2. The increased OATP expression and function in addition to increased ROS generation following MC exposure in aggregate culture relative to monolayer suggests a more realistic model for MC toxicity. The approach discussed here-in can not only be utilized to further probe into mechanisms of MCs' hepatocellular toxicity and markedly improve risk assessment, but to screen for OATP involvement in drug or toxin uptake and disposition in the liver, particularly important when evaluating elimination or drug interactions.

2. Methods & materials

2.1. Chemicals and reagents

HepG2, a human hepatocellular carcinoma derived cell line, was obtained from the ATCC (Manassas, VA). For cell culture and uptake studies, Dulbecco's Modified Eagle's Medium (DMEM) with pyruvate, Dulbecco's Phosphate Buffered Saline (DPBS) w/Calcium and Magnesium, Penicillin–Streptomycin (100×), heat inactivated Fetal Bovine Serum (FBS), and Hank's Buffered Saline Solution (HBSS) w/Calcium and Magnesium were purchased from Invitrogen Life Technologies (Grand Island, NY). Microcystin LR was obtained from VWR International through Enzo Life Sciences (Farmingdale, NY) and GreenWater Laboratories (Palatka, Florida) (fluorescent uptake and inhibition studies only).

For mask, wafer and PDMS mold generation, silicon prime wafers (P/Boron) were obtained from University Wafers (Boston, Massachusetts) and SU-8 Photoresist was purchased from MicroChem Corp. (Newton, MA) The Sylard 184 kit for polydimethylsiloxane (PDMS) was purchased from Dow Corning (Midland, MI). Master mask and chemicals for mask and wafer development, along with L-edit Tanner EDA software (Monrovia, CA) were obtained in kind from the Northern California Nanotechnology Center (NCNC, University of California, Davis).

For microwell fabrication, poly(ethylene glycol) diacrylate (PEGDA), average Mn 700, and 3-(trimethoxysilyl) propyl methacrylate (TMSPMA), sodium hydroxide solution, and 70% ethanol were purchased from Sigma Aldrich (St. Louis, MO), and glass slides were purchased from Fisher Scientific (Philadelphia, PA). The UV light source (Omnicure S1000) with filter (wavelength: 320–500 nm) was obtained from EXFO Photonic Solutions Inc (Ontario, Canada). The photo initiator Irgacure 2959 (4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone) was obtained from Ciba Specialty Chemicals, Switzerland. For mRNA expression studies, TRIZOL Reagent, SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase, RNase Away, RNase free water, TAE Buffer, Ethidium Bromide, DNA ladders and relevant primers (OATP1B1, OATP1B3 and GADPH) were ordered from Invitrogen (Carlsbad, CA). Chloroform, isopropyl alcohol and ethanol were obtained from Sigma Aldrich (St. Louis, MO).

For fluorescence uptake and inhibition studies, 8-FcA, (8-2-[Fluoresceinyl]-aminoethylthio) adenosine-3′, 5′-cyclic monophosphate, was purchased from Biolog Life Science Institute (Bremen, Germany) through its North American distributor, Axxora, LLC (San Diego, CA) and fluorescein-methotrexate (FMTX) was purchased from Invitrogen (Carlsbad, CA). For the inhibition studies, Rifampicin was purchased from Sigma Aldrich (St. Louis, MO), deoxycholic acid and cyclosporine D were purchased from Calbiochemicals (Los Angeles, CA), and microcystin LR