Cell culture

Assessment of cancer spheroid formation in Human Plasma-Like Medium

Keywords
Cancer, 3D cell culture, spheroids, physiology, drug screening, cell health

Summary

- Human Plasma-Like Medium (HPLM) is a cell growth formulation designed to mimic the metabolic profile of human plasma to help maintain the physiological state of cells.
- We demonstrate the capability of HPLM to support the growth of cancer spheroids in several well-studied cancer cell lines.

Introduction

3D cancer spheroid models represent *in vivo* tumor-like environments more accurately than standard 2D models and may help provide more relevant results in cancer drug screening [1,2]. Standard cell culture media supplement these spheroids with nutrients at concentrations that differ from *in vivo* nutrient availability. To help minimize discrepancies between *in vitro* and *in vivo* findings, it is necessary to choose culture conditions that are physiologically relevant [3-5].

Gibco™ Human Plasma-Like Medium (HPLM) addresses the need for physiologically relevant culture conditions by recapitulating the composition of metabolites in human plasma. Research has shown HPLM to be similar to the natural human cellular environment. *In vitro* culture of various cancer cells using HPLM demonstrated an increase in *de novo* pyrimidine synthesis in the cells, which resulted in more resistance to the DNA-damaging effects of the chemotherapeutic agent 5-fluorouracil [6]. Compared to a standard medium, HPLM also supported improved activation of human T cells upon antigen stimulation [7]. Here we assessed the formation of cancer spheroids using HPLM compared to standard media. Regular and dialyzed Gibco™ Fetal Bovine Serum (rFBS and dFBS, respectively) were included with HPLM so that the appropriate FBS to use along with these media could be assessed. Multiple tumor cell lines were used to generate scaffold-free 3D spheroids in various standard basal media as well as in HPLM. Additionally, these spheroids were assessed for sensitivity to standard care chemotherapeutic agents.

We tested eight commonly used cancer cell lines (A431, A549, HCT116, HT1080, PANC-1, PC-3, T470, and U87MG) belonging to different cancer types. We observed that most of the eight cell lines adapted well to HPLM and were generally able to form spheroids without requiring any additional supplements. PC-3 cells, which need extracellular matrix (ECM) protein supplementation to form robust spheroids in standard basal medium, did require ECM supplementation for spheroid culture in HPLM as well. By supplementing HPLM with either regular or dialyzed FBS, we observed that both medium and serum played a role in spheroid formation, cell health, and response to cytotoxic drugs. Taken together, our results demonstrate that HPLM supports spheroid formation from multiple cancer cell types, while cell health and response to cytotoxic drugs differ from cells grown in HPLM versus standard culture medium.

Results

Comparison of spheroid morphology and size between HPLM and standard medium

Eight human cancer cell lines were tested for their ability to form reproducible 3D spheroids *in vitro* in both standard basal media (Gibco™ RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES) and HPLM. HPLM was supplemented with either 10% rFBS or 10% dFBS and RPMI medium was supplemented with 10% rFBS. All cells were cultured in the three media conditions for 3 passages (according to ATCC recommended protocol) prior to spheroid formation. Freely floating spheroids were generated on Thermo Scientific™ Nunclon™ Sphera™ 96-Well Microplates as one spheroid per well using laboratory-developed protocols [8]. Figure 1 shows representative spheroids from T47D, HCT116, A549, U87MG, and PANC-1 cells. Most of the spheroids formed compact spherical structures, which increased in size over time in all culture conditions. However, PANC-1 cells grown in HPLM did not form compact 3D structures as they did in RPMI standard medium. PANC-1 spheroids were irregularly shaped, especially when in HPLM supplemented with dFBS. Additionally, as shown in Figure 1, T47D spheroids in HPLM were smaller than those in RPMI standard medium. This can be attributed to the reduced growth rate of T47D cells in HPLM.

Compactness of spheroids

Spheroids formed in HPLM and standard media were checked for compactness by staining with Invitrogen™ Vybrant™ DiI Cell-Labeling Solution (1 µM in Gibco™ CTS™ DPBS for 2 hours) per the manufacturer's instructions. A compact spheroid limits entry of the dye toward its core, resulting in a ring-like staining pattern. As shown in Figure 2, T47D spheroids formed in all media conditions were equally compact. Figure 2 also shows PANC-1 spheroids, for which there is a difference in compactness between those grown in HPLM and those grown in standard media (Gibco™ DMEM, high glucose, GlutaMAX™ Supplement). Comparatively, spheroids grown in DMEM standard media supplemented with rFBS and in HPLM supplemented with rFBS demonstrated similar compactness, while those grown in HPLM supplemented with dFBS did not form compact spheroids.

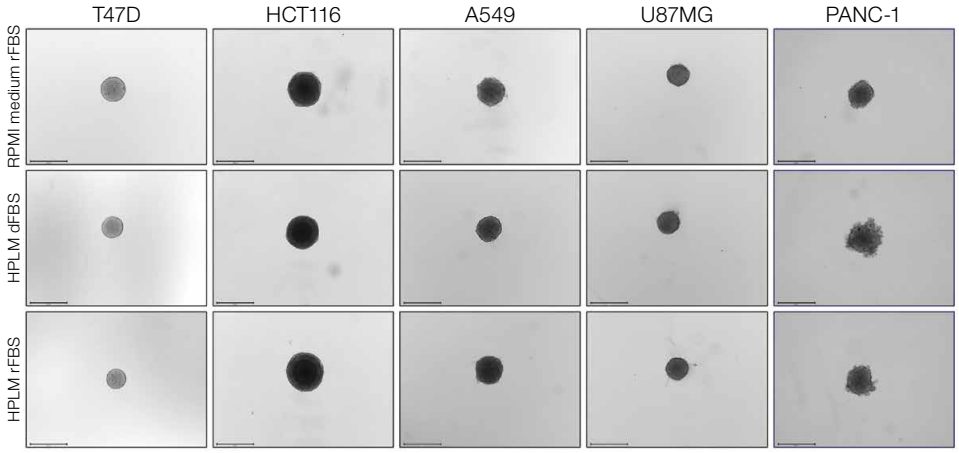


Figure 1. HPLM supports 3D spheroid formation. Representative images of spheroids from various cell types cultured in RPMI standard medium and HPLM that were grown for 5 days. Images were captured using the Invitrogen™ EVOS™ M7000 Imaging System. Scale bar = 650 µm.

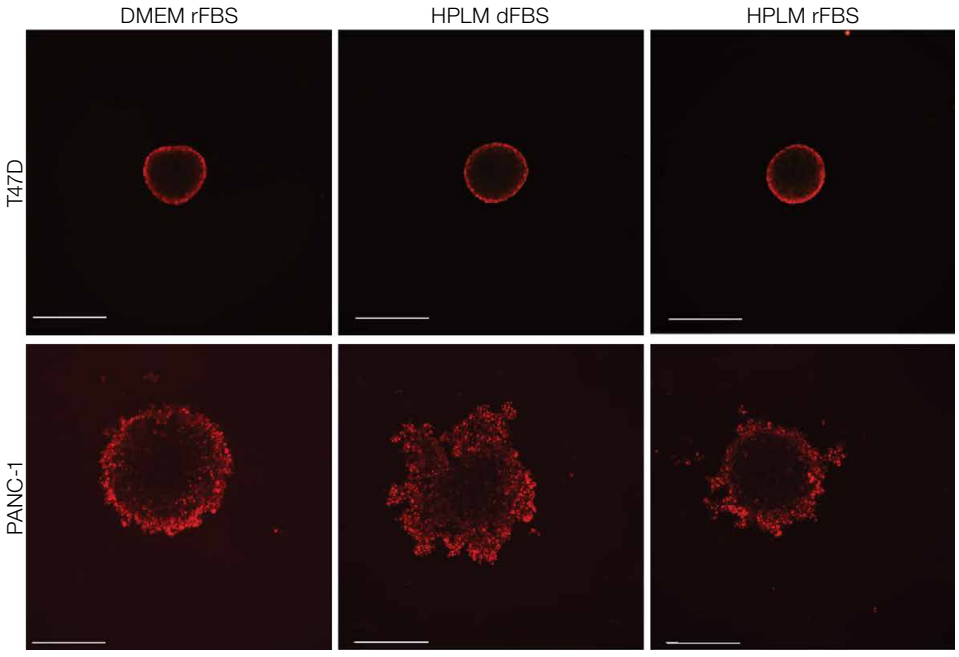


Figure 2. Assessment of spheroid compactness. 6-day-old spheroids from T47D cells (4,000 cells/spheroid) and PANC-1 cells (5,000 cells/spheroid) were stained with Vybrant DiI Cell-Labeling Solution. Images were captured using the Thermo Scientific™ CellInsight™ CX7 LZR High Content Analysis Platform under a 4x objective. Scale bar = 400 µm.

ECM requirement for spheroids

PC-3 cells cultured in standard growth medium require the addition of ECM protein (Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix) to the medium for spheroid formation. In the absence of Geltrex matrix, loose cell aggregates are formed (Figure 3A, day 4). Addition of 3% Geltrex matrix in DMEM medium supplemented with rFBS resulted in well-defined and round-shaped spheroids (Figure 3A), as also confirmed by staining with Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (Hoechst 33342) (Figure 3B). In contrast, HPLM supplemented with rFBS or dFBS did not support the growth of compact spheroids even in the presence of Geltrex matrix (Figure 3A). Figure 3B shows the decreased compactness and irregular borders of spheroids grown in HPLM. This result indicates that HPLM is not an ideal choice for growing PC-3 spheroids.

Cell health of 3D spheroids in HPLM

Following spheroid formation, the health and viability of the cells were assessed. The Promega™ CellTiter-Glo™ Assay was used to provide an indirect measure of cell viability. This assay measures production of ATP, which correlates to mitochondrial metabolism and thus cell viability, growth, or health. We observed a cell type–dependent result for this assay. For example, in T47D spheroids, the ATP production correlated to spheroid size (Figure 4A), indicating slower growth of cells in HPLM compared to RPMI medium. For HCT116, even though spheroid size was equivalent in all 3 media conditions, ATP production was reduced in HPLM compared to Gibco™ McCoy's 5A (Modified) Medium (Figure 4B). For A431 spheroids, ATP production in HPLM supplemented with rFBS was almost 50% less when compared to DMEM supplemented with rFBS (Figure 4C). This can be attributed to the slower growth of cells in monolayer in HPLM with rFBS. Interestingly, even though the growth of A431 cells in monolayer was slowest in HPLM supplemented with dFBS (data not shown), ATP production in these spheroids was comparable to that in DMEM with rFBS. This result indicates increased mitochondrial metabolism or growth of cells in this medium in a 3D format. Further confirmatory studies would help in understanding the molecular mechanisms underlying such a response.

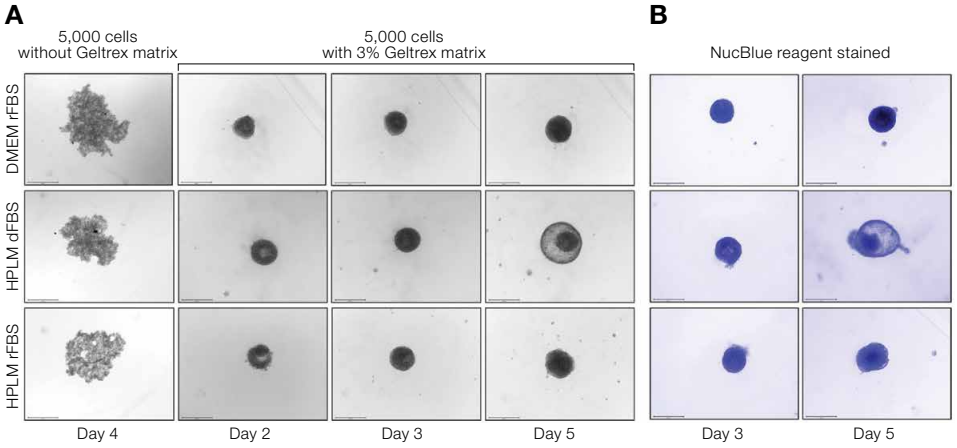


Figure 3. Representative images of spheroids from PC-3 cells in HPLM and standard medium. (A) 5,000 cells were seeded per well with and without Geltrex matrix in different media conditions. Images were captured at different time points using the EVOS M7000 Imaging System. Scale bar = 650 µm. **(B)** PC-3 spheroids grown in Geltrex matrix–containing medium were stained using NucBlue Live Ready Probes reagent (2 drops per milliliter of medium) and images were captured using the EVOS M7000 Imaging System (DAPI brightfield filter). Scale bar = 650 µm.

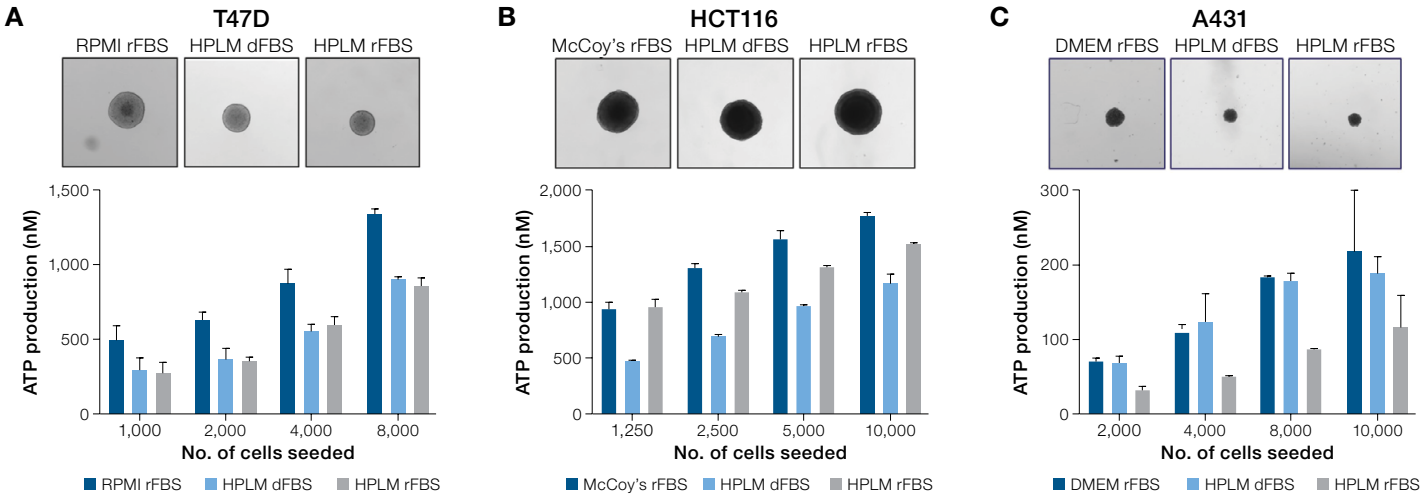


Figure 4. Assessment of cell viability in spheroids. Total intracellular ATP content measured by luminescence using the CellTiter-Glo Assay in **(A)** T47D, **(B)** HCT116, and **(C)** A431 spheroids grown in different media conditions. Readings were obtained using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader. Error bars represent standard deviation.

Imaging-based assessment of cell health

To obtain a direct visualization of cell health, spheroids grown in all media conditions were stained with the Invitrogen™ LIVE/DEAD™ Viability/Cytotoxicity Kit. This kit consists of calcein AM and ethidium homodimer, which stain live and dead cells, respectively. Live and dead cell populations appeared similar across all media conditions in all cell types tested (Figure 5A). Mitochondrial superoxide release, a response marker for oxidative stress, was compared between the media conditions to assess if culturing in HPLM induces cellular stress. Invitrogen™ MitoSOX™ Red Mitochondrial Superoxide Indicator was added to the spheroids at a concentration of 2 µM, followed by incubation at 37ºC for 4 hours. After washing three times in DPBS containing calcium and magnesium, the spheroids were imaged using the CellInsight CX7 LZR imaging system. As a positive control for visualizing mitochondrial superoxide release (Figure 5B, positive control), spheroids were serum-starved for 48 hours, followed by exposure to 100 µJ/cm^2 of UV light for 1 hour before staining. No noticeable mitochondrial superoxide release was observed, indicating that HPLM does not induce oxidative stress in spheroids (Figure 5B).

We made contrasting observations in two of the cell lines that were tested. HT1080 cells are normally propagated in DMEM supplemented with 10% FBS. When grown in HPLM, these cells exhibited more elongated morphology, indicative of cellular stress (Figure 6A). To further assess this finding, monolayer cells grown in different media conditions were labeled with MitoSOX Red Mitochondrial Superoxide Indicator (750 nM). The mitochondria of HPLM–grown cells exhibited more red fluorescence, indicating the presence of superoxide, whereas the mitochondria of cells in standard media showed minimal fluorescence (Figure 6B). Regardless of their morphology in monolayer, these cells formed compact spheroids in all the media conditions (Figure 6C). A similar observation was also made for HCT116 cells. Regardless of the slower growth of monolayer in HPLM compared to standard medium (Figure 6D), spheroids formed in all media conditions were equivalent in size (Figure 6E). However, for HT1080 spheroids, the basal level of caspase activation was higher in spheroids grown in HPLM, indicative of cellular stress (Figure 6F).

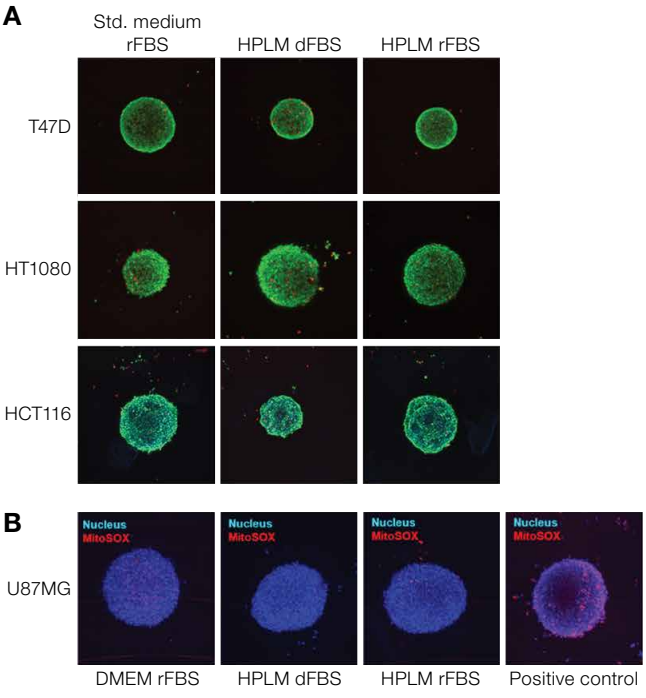


Figure 5. Visualization of cell health in spheroids. (A) T47D, HT1080, and HCT116 spheroids were stained with the LIVE/DEAD Viability/Cytotoxicity Kit reagents and **(B)** U87MG spheroids were stained with MitoSOX Red Mitochondrial Superoxide Indicator. Nuclei were stained using NucBlue Live ReadyProbes Reagent at 2 drops per milliliter of medium. Images were acquired using the CellInsight CX7 LZR imaging system under a 4x objective.

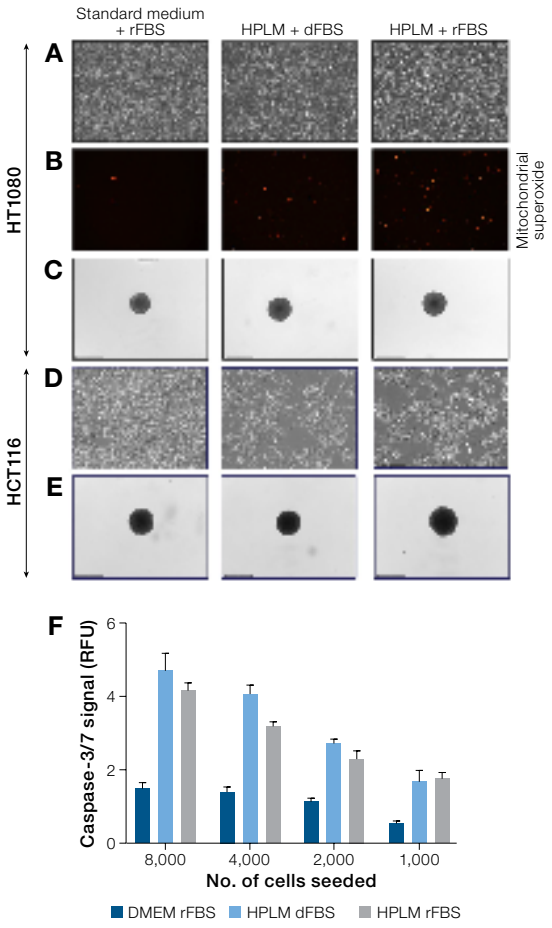


Figure 6. Assessment of cell health. (A) Representative images of HT1080 cells cultured in HPLM or standard media for 5 days. Scale bar = 275 µm. **(B)** HT1080 monolayers in different media conditions were stained with MitoSOX Mitochondrial Superoxide Indicator reagent. Red fluorescence indicates superoxide release. **(C)** Morphology of HT1080 spheroids grown in different media conditions. Scale bar = 650 µm. **(D)** HCT116 monolayer cells in different media conditions. **(E)** Morphology of HCT116 spheroids grown in different media conditions. Scale bar = 650 µm. All images were captured using the EVOS M7000 Imaging System. **(F)** 5-day-old HT1080 spheroids were stained with Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent (4 µM) and incubated overnight. Fluorescence was measured using the Varioskan LUX Multimode Microplate Reader at Ex/Em 502/530 nm.

Response to cytotoxic drugs in 3D cultures

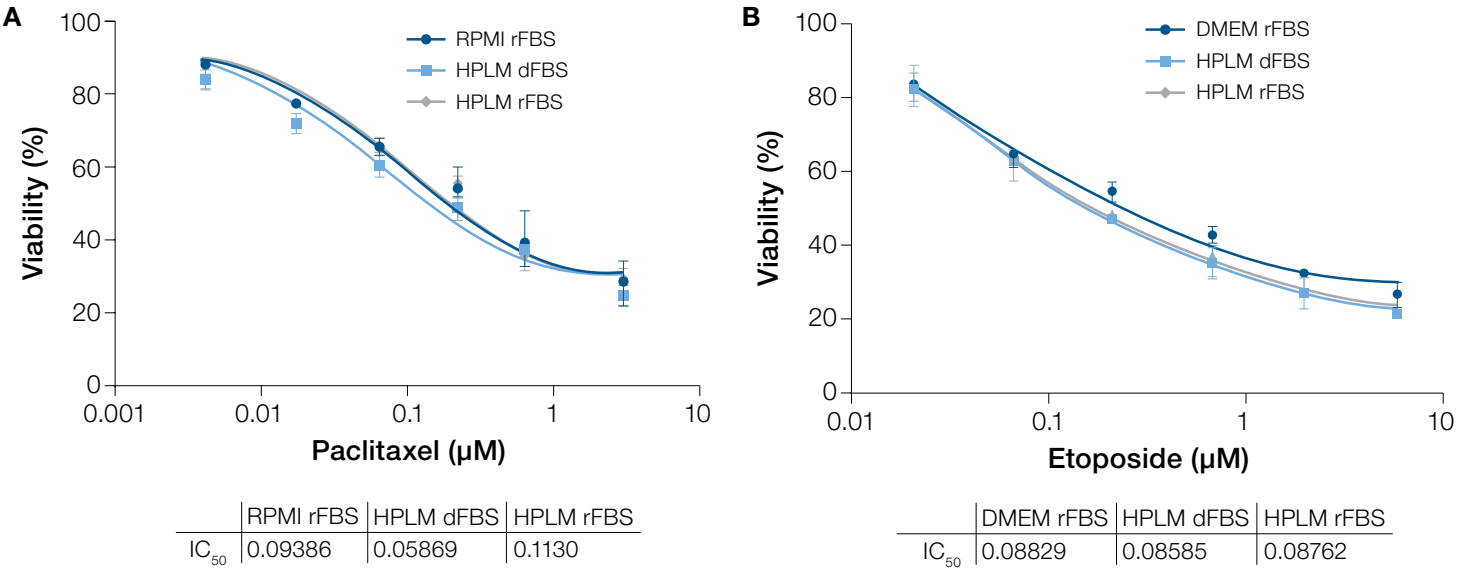
To determine if a change in drug sensitivity occurs when cells are cultured in HPLM rather than standard media, we compared responses to the cytotoxic drugs paclitaxel, etoposide, 5-fluorouracil, and staurosporine. The results indicate variation in drug responses among cell types; some cells showed an equivalent response, while others showed increased or reduced sensitivity to the drugs.

Equivalent response

3D spheroids from T47D, HT1080, U87MG, and A549 cells grown in all media conditions were subjected to cytotoxic treatment with paclitaxel, etoposide, 5-fluorouracil, and staurosporine (Table 1). The cells responded equivalently to the drugs, as indicated by similar IC_{50} values between the media conditions for paclitaxel and etoposide (Figure 7).

Table 1. Parameters for drug sensitivity assessment in HPLM and standard media.

Cell line	Chemotherapeutic drug	Concentration	Duration of treatment
T47D	Paclitaxel	Starting = 1.6 µM 1:3 dilution 5 dilutions	72 hours
HT1080	Etoposide	Starting = 6.6 µM 1:3 dilution 6 dilutions	72 hours
U87MG	5-Fluorouracil	Starting = 4 mM 1:3 dilution 11 dilutions	144 hours
A549	Staurosporine	Starting = 50 µM 1:3 dilution 11 dilutions	96 hours



	RPMI rFBS	HPLM dFBS	HPLM rFBS
IC_{50}	0.09386	0.05869	0.1130

	DMEM rFBS	HPLM dFBS	HPLM rFBS
IC_{50}	0.08829	0.08585	0.08762

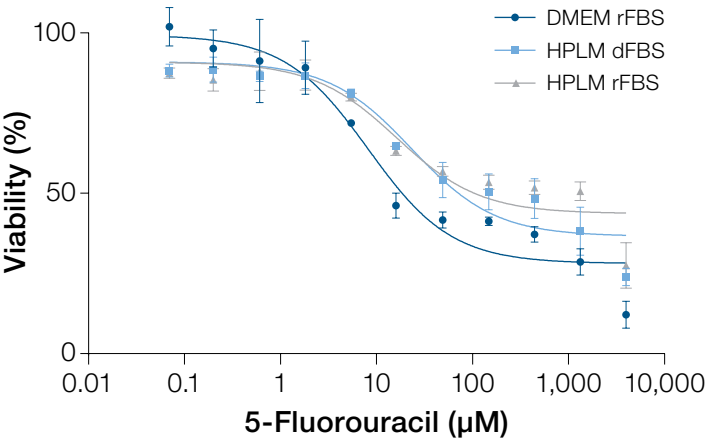
Figure 7. Dose response curves obtained for each drug administered at 72 hours. Viability of **(A)** T47D spheroids treated with paclitaxel and **(B)** HT1080 spheroids treated with etoposide was determined using Invitrogen™ PrestoBlue™ HS Cell Viability Reagent; readings were obtained with the Varioskan LUX Multimode Microplate Reader at Ex/Em 560/590 nm. Error bars represent standard error of the mean; n = 2.

Increased drug resistance

Interestingly, we noticed a difference in 5-fluorouracil (5-FU) drug sensitivity in 3D cultures of U87MG cells grown in different media conditions. Based on the dose response curves, IC_{50} values increased to approximately 2 times and 3 times the initial values in HPLM rFBS and HPLM dFBS, respectively, compared to DMEM standard medium (Figure 8). This indicates that U87MG spheroids in HPLM are more resistant to 5-FU–mediated cytotoxicity. Previous studies have also reported similar findings for other cell types [5].

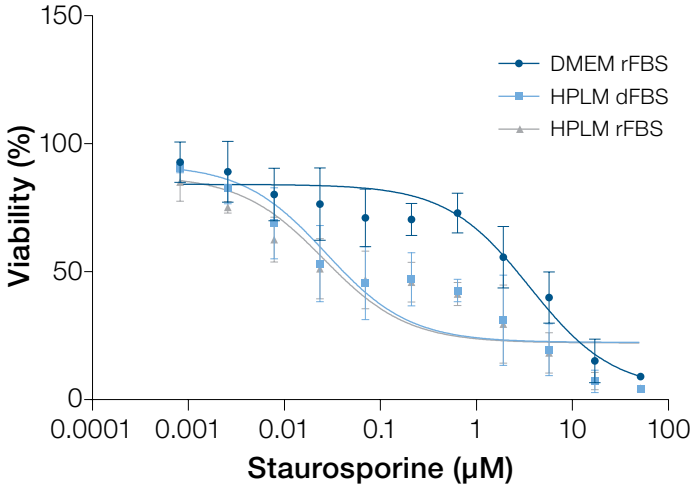
Increased drug sensitivity

A549 spheroids grown for over 15 doublings in HPLM showed increased sensitivity to staurosporine-mediated cytotoxicity (Figure 9). The IC_{50} of staurosporine for spheroids grown in HPLM (both rFBS and dFBS conditions) was approximately 100 times lower than that for spheroids grown in DMEM medium containing rFBS. Further analysis of cell health and molecular changes within the cells in these media would help in understanding the difference in response.



	DMEM rFBS	HPLM dFBS	HPLM rFBS
IC_{50}	8.240	23.47	16.29

Figure 8. Viability of U87MG spheroids treated with 5-FU for 6 days. Cells were cultured in HPLM and standard medium (DMEM). Cell viability was assessed by PrestoBlue HS Cell Viability Reagent readings, obtained using the Varioskan LUX Multimode Plate Reader at Ex/Em 560/590 nm. Error bars represent standard error of the mean; n = 3.



	DMEM rFBS	HPLM dFBS	HPLM rFBS
IC_{50}	3.504	0.02646	0.02512

Figure 9. Viability of A549 spheroids treated with staurosporine for 96 hours. Cells were grown in HPLM and standard medium (DMEM). Cell viability was assessed by PrestoBlue HS Cell Viability Reagent readings, obtained using the Varioskan LUX Multimode Microplate Reader at Ex/Em 560/590 nm. Error bars represent standard error of the mean; n = 3.

Conclusion

Taken together, our results demonstrate that the compatibility of HPLM is cell-line specific and spheroids formed using this medium are robust, reproducible, and suitable for drug efficacy studies. We were able to show that spheroid formation and cell health in HPLM are comparable to spheroid formation and cell health in standard media with a few exceptions. Further studies are needed to explore such reasons. By using physiologically relevant culture media, researchers could be able to comprehend the nutritional environment of tumors and, thus, design better cancer models.

References

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8. thermofisher.com/in/en/home/references/protocols/cell-culture/3-d-cell-culture-protocol.html

Note: For protocols on spheroid formation, refer to the protocol here.

Ordering information

Product	Cat. No.
Cell culture plastics	
Nunclon Sphera 96-Well, Nunclon Sphera-Treated, U-Shaped-Bottom Microplate	174925
Cells, media, ECM products, and supplements	
DMEM, high glucose, GlutaMAX Supplement	10566016
RPMI 1640 Medium, GlutaMAX Supplement, HEPES	72400047
Human Plasma-Like Medium (HPLM)	A4899102
Fetal Bovine Serum (FBS)	A5256701
Penicillin-Streptomycin (10,000 U/mL)	15140122
Fetal Bovine Serum, dialyzed, US origin, One Shot format	A3382001
CTS DPBS, calcium, magnesium	A1285801
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	A1413201
Dyes and kits	
Vybrant DiI Cell-Labeling Solution	V22885
CellEvent Caspase-3/7 Green Detection Reagent	C10423
LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells	L3224
CellTiter-Glo 3D Cell Viability Assay (Promega Corporation)	G7571
MitoSOX Red Mitochondrial Superoxide Indicator, for live-cell imaging	M36008
NucBlue Live ReadyProbes Reagent (Hoechst 33342)	R37605
Instruments	
Varioskan LUX Multimode Microplate Reader	VL0000D0
EVOS M7000 Imaging System	AMF7000
CellInsight CX7 LZR High Content Analysis Platform	CX7A1110LZR

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