Supporting Information

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Assessment of Hepatotoxic Potential of Cyanobacterial Toxins Using 3D In Vitro Model of Adult Human Liver Stem Cells

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Supporting Information

Methods

Cell culture

HL1-hT1 cells were routinely cultured in Keratinocyte-SFM medium with 5 ng/mL recombinant human epidermal growth factor and 50 µg/mL bovine pituitary extract (Gibco by ThermoFisher, Waltham, MA), and further supplemented with 2 mM of N-Acetyl-L-cysteine, 5 mM of nicotinamide, 0.2 mM of ascorbic acid (all Sigma-Aldrich, St. Louis, MO) and 10 % fetal bovine serum (Biochrom, Cat. No. S0615, Berlin, Germany). All incubations and exposures of HL1-hT1 cells were carried out at 37 °C in a cell culture incubator at 95% relative humidity with a 5% CO_2 supply. Cells were routinely cultured in T25 flasks (TPP, Trasadingen, Switzerland) to ~80% confluence and passaged in 1:10 ratio using Trypsin/EDTA (Gibco) once per week.

Scaffold-free spheroid cultures were prepared in agarose hydrogels, using sterile 3D Petri Dish® micromolds (type 24-35, Sigma-Aldrich). 330 µL of 2% (w/v) sterile molten (60°C) agarose (Sigma-Aldrich, Cat. No. A9539) dissolved in a 0.9 % (w/v) sodium chloride was pipetted into a micromold for casting gels fitting 24-well microplates. Solidified micromolded gels were transferred into the wells of 24-well microplate (TPP), and then immobilized with 120 µL of molten agarose (39°C) pipetted into each well outside the gel. Immobilized gels were then equilibrated twice for 15 min with 1 mL of complete culture media. Cell suspension (75 µL) was seeded subsequently in the inner chamber of the gel containing 35 microwells (800 µm diameter x 800 µm depth). The cells were allowed to settle into the microwells for 10 min, then 1 mL of cell culture or exposure media was added to each well. In all spheroid experiments, cell culture or exposure medium (1 mL) was exchanged after 96 h, 168 h and 264 h of culture.

Cell viability

Monolayer: HL1-hT1 cells were seeded 48 h prior the exposure at the density 18×10^3 cell/cm^2 into 96-well plates (100 µL per well), which corresponded to 3.6×10^3 cell/well for the impedimetric experiments conducted in E-plates (ACEA Biosciences, San Diego, CA) with 0.2 cm^2 well bottoms, and to 6×10^3 cell/well for viability assays conducted in traditional tissue-culture treated polystyrene microplates (Greiner Bio One, Kremsmunster, Austria) with 0.34 cm^2 well bottom area.

Cell viability was also evaluated, using a combination of three metabolic assays, evaluating cell respiration and dehydrogenase activity (Alamar Blue, AB), esterase activity and membrane integrity (5-Carboxyfluorescein Diacetate-Acetoxymethyl Ester, CFDA-AM), and uptake and accumulation of Neutral Red (NR) dye [1]. The assays were conducted with the cells seeded into polystyrene 96-well black plates (Greiner Bio One, Kremsmunster, Austria). Following 48 h or 96 h exposure, the cells were washed with phosphate-buffered saline (PBS) and incubated with a serum-free medium containing 4% (v/v) AB and 4 µM CFDA-AM (both ThermoFisher). After 30 min of incubation, the fluorescence of AB (excitation/emission: 530/590 nm) and CFDA-AM (excitation/emission: 485/520 nm) was measured by a microplate reader Synergy 4 Reader (BioTek, Winooski,

VT). The cells were then rinsed again with PBS and incubated for 2 h with NR (0.005%, w/v) dissolved in a serum-free medium. After the incubation, the cells were rinsed again with PBS and NR was extracted by 1% (v/v) acetic acid in 50% (v/v) ethanol. NR absorbance was measured at 540 nm wavelength (690 nm reference) by the microplate reader. Absorbance/fluorescence from the blank wells incubated with AB/CFDA-AM/NR, but without the cells, were subtracted from the readouts of the experimental wells. Blank-subtracted values were compared with the nontreated control and expressed as a % of control. All experimental conditions were prepared in triplicates and the experiments were repeated at least three times independently.

Spheroid cultures: The exposure medium (1 mL) was aspirated from the outside of the agarose gel, which was rinsed twice for 15 min with 1 mL of serum-free culture medium. Spheroids were then incubated for 4 h with 1 mL of AB (4%, v/v, in serum-free culture medium). Microplates were then gently shaken on the orbital shaker (10 min), and AB solution from each experimental well was pipetted in triplicate (3x250 µL) into the 96-well plate (Greiner). Fluorescence of AB was measured as described above. Wells with the agarose gel, but without the spheroids, were used as a blank, and the blank-subtracted readouts from the experimental treatments were then compared with the nontreated control and expressed as a % of control. Each treatment (gel with 35 spheroids) was repeated at least in three independent experiments.

LC-MS/MS analysis of microcystin-LR (MC-LR) and cylindrospermopsin (CYN)

Total concentrations of MC-LR and CYN in the exposure medium during the *in vitro* experiments were determined by LC-MS/MS. Exposure medium was collected from the wells with the monolayer cultures (t=24, 48, 72 and 96 h of exposure) or spheroid cultures (t=96, 168, 264 and 336 h of exposure, when the medium was exchanged/removed, and also 1 h after addition of fresh exposure media, i.e. t=1, 97, 169 and 265 h). As a control, cyanotoxins were analysed also in the parallel blank wells containing exposure medium without the cells. Culture medium was aspirated after a given exposure period into microcentrifuge tubes, preserved by addition of an equal volume of 100% MeOH and stored at −20°C. Before analysis, the samples were clarified by centrifugation (20,000×G, 15 min) and filtered via 0.45 µm Nylon syringe filter (Labicom, Olomouc, Czech Republic).

Concentration of MC-LR was determined by Waters Acquity UPLC using Waters Aqcuity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) with a 2.1 x 5 mm pre-column (Waters, Dublin, Ireland). Mobile phase consisted of 0.1% formic acid (A) and 0.1% formic acid- acetonitrile (B). Gradient elution started with 20%B at 0 min and ramped to 60% at 5 min and to 90% at 5.5 min (flow rate 0.4 mL/min, temperature 30°C, injection volume 5 µL). After 8 min, the column was equilibrated for 2.5 min with 20%B. Waters Xevo TQS was used in the positive mode of electrospray ionization (ESI): capillary voltage 3500 V, drying gas temperature 350°C, flow rate 800 L/h, pressure 7 bar, collision energy 60 V. Multiple reaction monitoring (MRM) was used to detect MC-LR transitions at m/z 996.5 > 135 and 213.1. External calibrations were used, with the instrumental limit of detection being 0.5 ng/mL (0.5 nM) and limit of quantification being 1 ng/mL (1 nM).

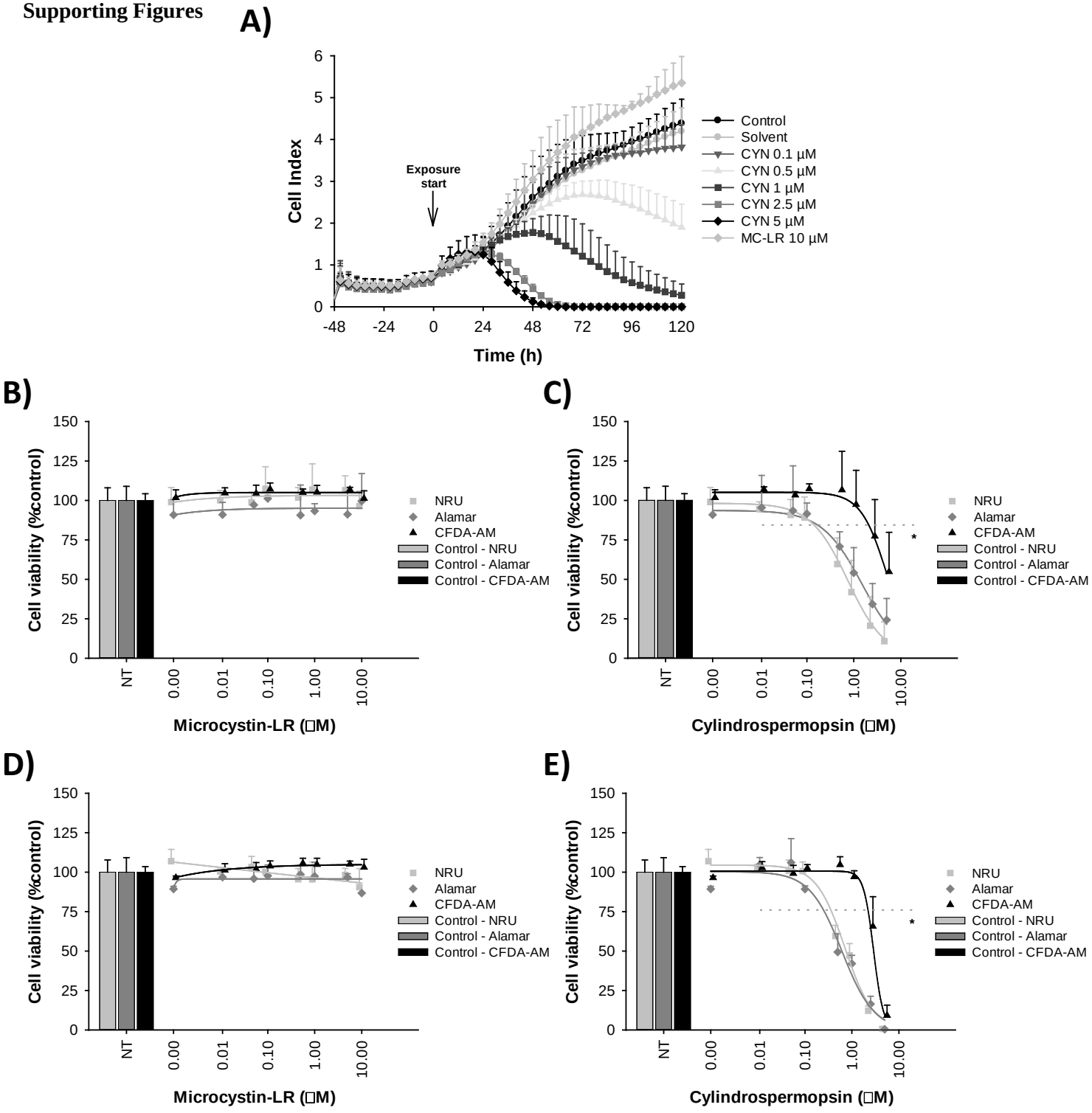
Concentration of CYN was analyzed by Agilent 1260 Infinity using Poroshell 120 EC-18 column (2.1 x 100 mm, 2.7 µm) with a 2.1 x 5 mm pre-column (Agilent Technologies, Waldbronn, Germany). Mobile phase consisted of

0.1% formic acid-1% acetonitrile (A) and 0.1% formic acid-acetonitrile (B). Elution started with of 0%B between 0-2.5 min which ramped to 50% at 2.6 min and was maintained for 1.4 min (flow rate 0.4 mL/min, temperature 35°C, injection volume 5 µL). The column was equilibrated for 6 min with 100%A. Agilent 6460 triple quadrupole was used in the positive mode of ESI: capillary voltage 4000 V, drying gas temperature 300°C, flow rate 300 L/h, nebulizing gas pressure 3 bar, jet voltage 500 V, collision energy 4 V. MRM was used to detect CYN transitions at m/z 416 > 194 and 179. External calibration was used, with the instrumental limit of detection being 0.15 ng/mL (0.4 nM) and limit of quantification being 0.4 ng/mL (1 nM).

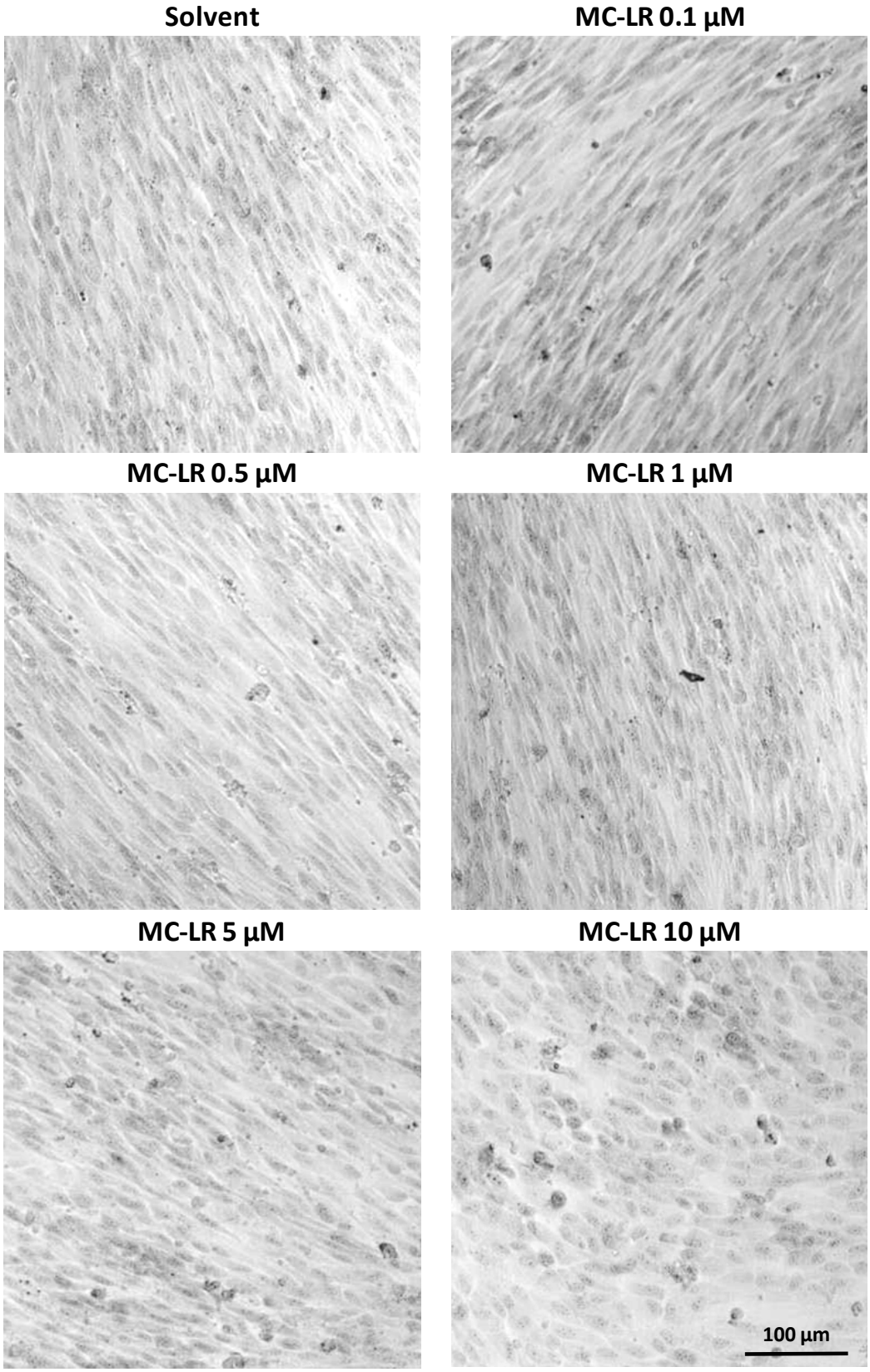
References

(1) Boaru, D. A.; Dragoş, N.; Schirmer, K.; Dragos, N.; Schirmer, K. Microcystin-LR Induced Cellular Effects in Mammalian and Fish Primary Hepatocyte Cultures and Cell Lines: A Comparative Study. *Toxicology* **2006**, *218* (2–3), 134–148.

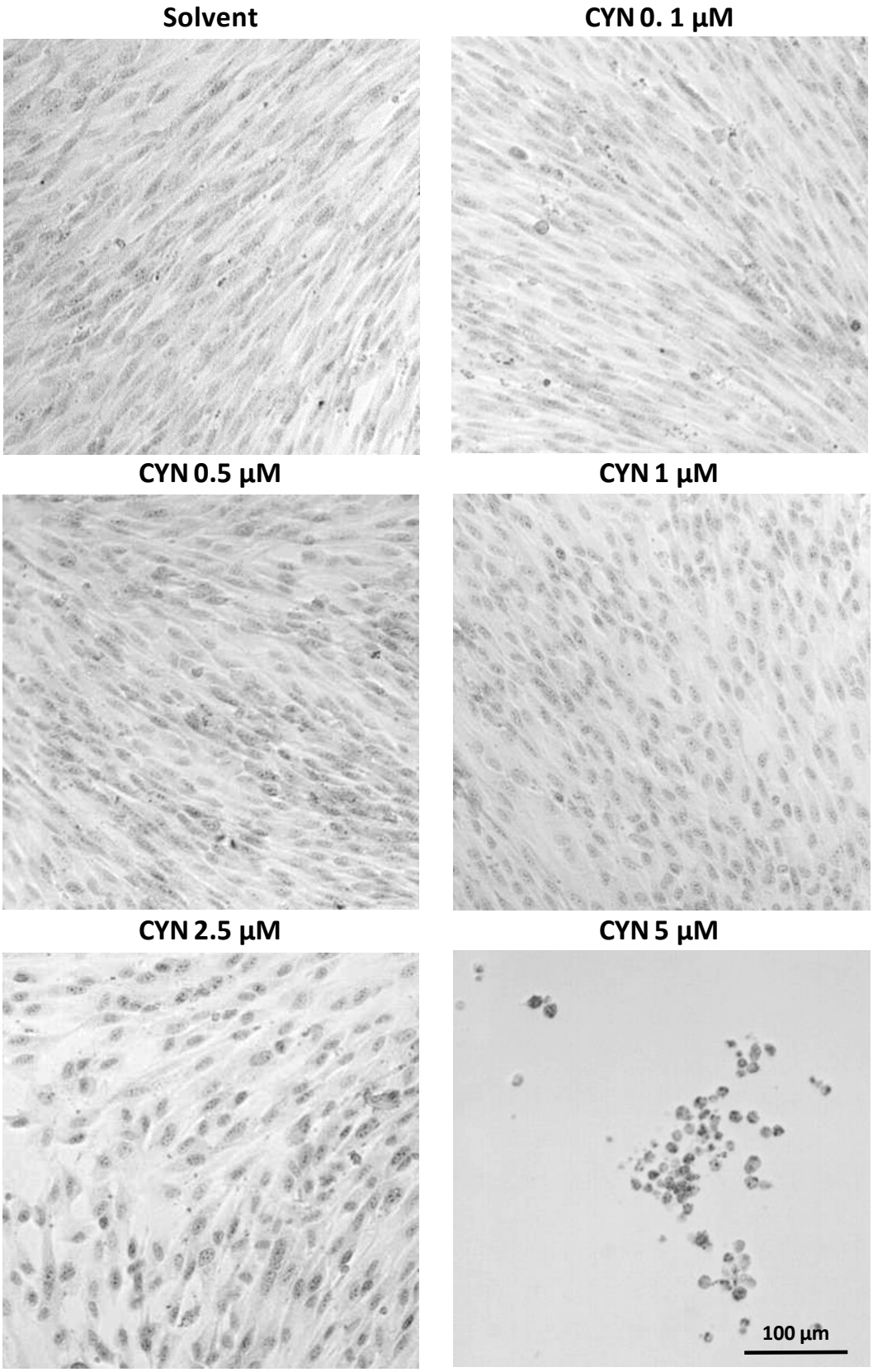
Supporting Figures



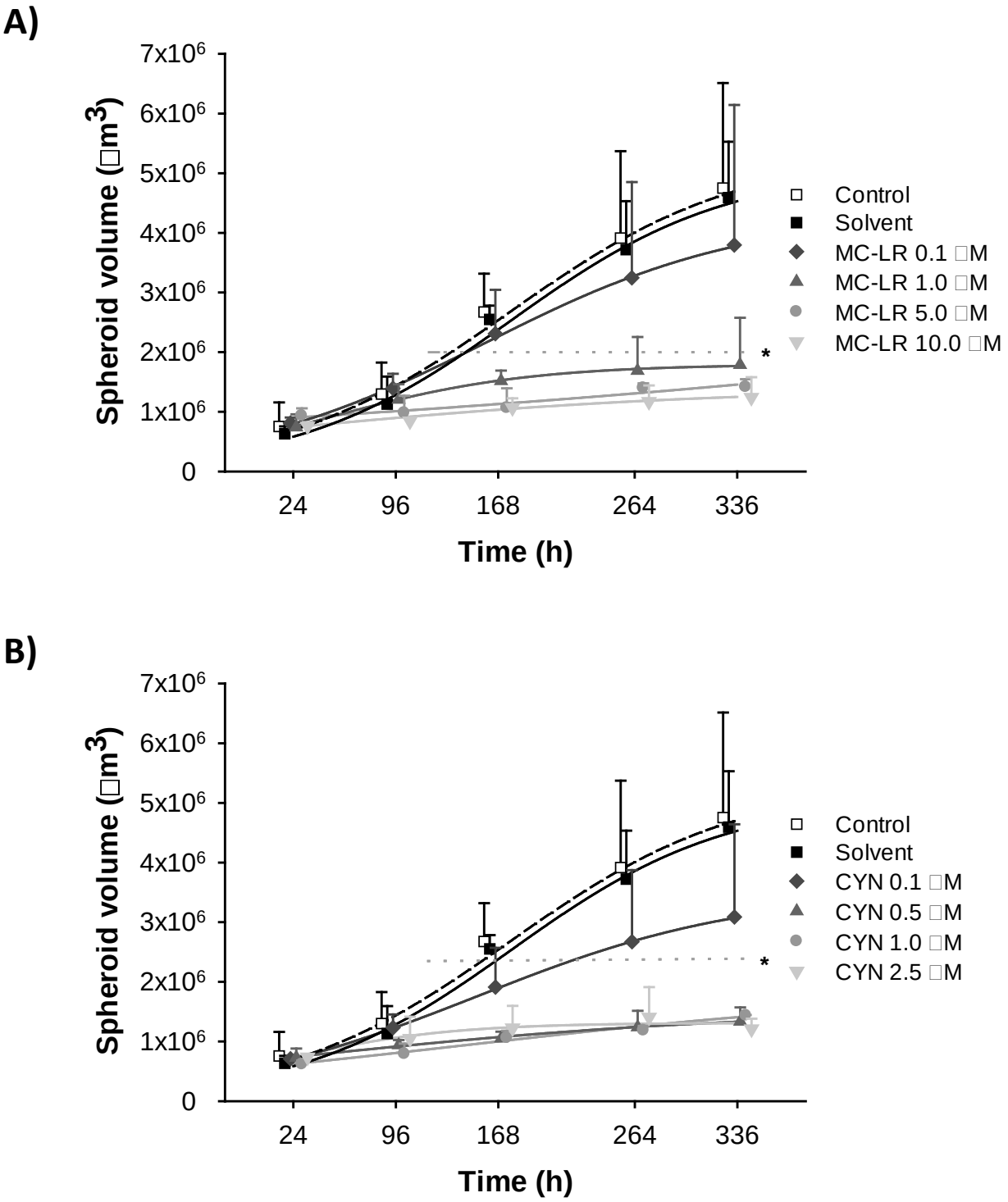
Supporting Figure S1. Effects of microcystin-LR (MC-LR) cylindrospermopsin (CYN) on the growth and viability of adult human liver stem cells in monolayer experiments. HL1-hT1 cells were treated 48 h post-seeding by cyanotoxin MC-LR or CYN. (A) Cell growth and condition was monitored for 120 h of exposure by real-time impedimetric analysis with impedance values reported as Cell Index. (B-E) Cell viability was evaluated by Neutral Red Uptake (NRU), Alamar Blue and CFDA-AM assays conducted after 48 h (B, C) or 96 h (D, E) of exposure. NT - nontreated control (Control), Solvent – solvent control (0.2% MeOH, v/v). Data represent average±SD values from independently repeated experiments, the curves in the graphs B-E represent a fit of 3-parameter sigmoid function. *Data points below the dashed line were significantly lower (Mann-Whitney test, $p<0.05$) than the solvent control.



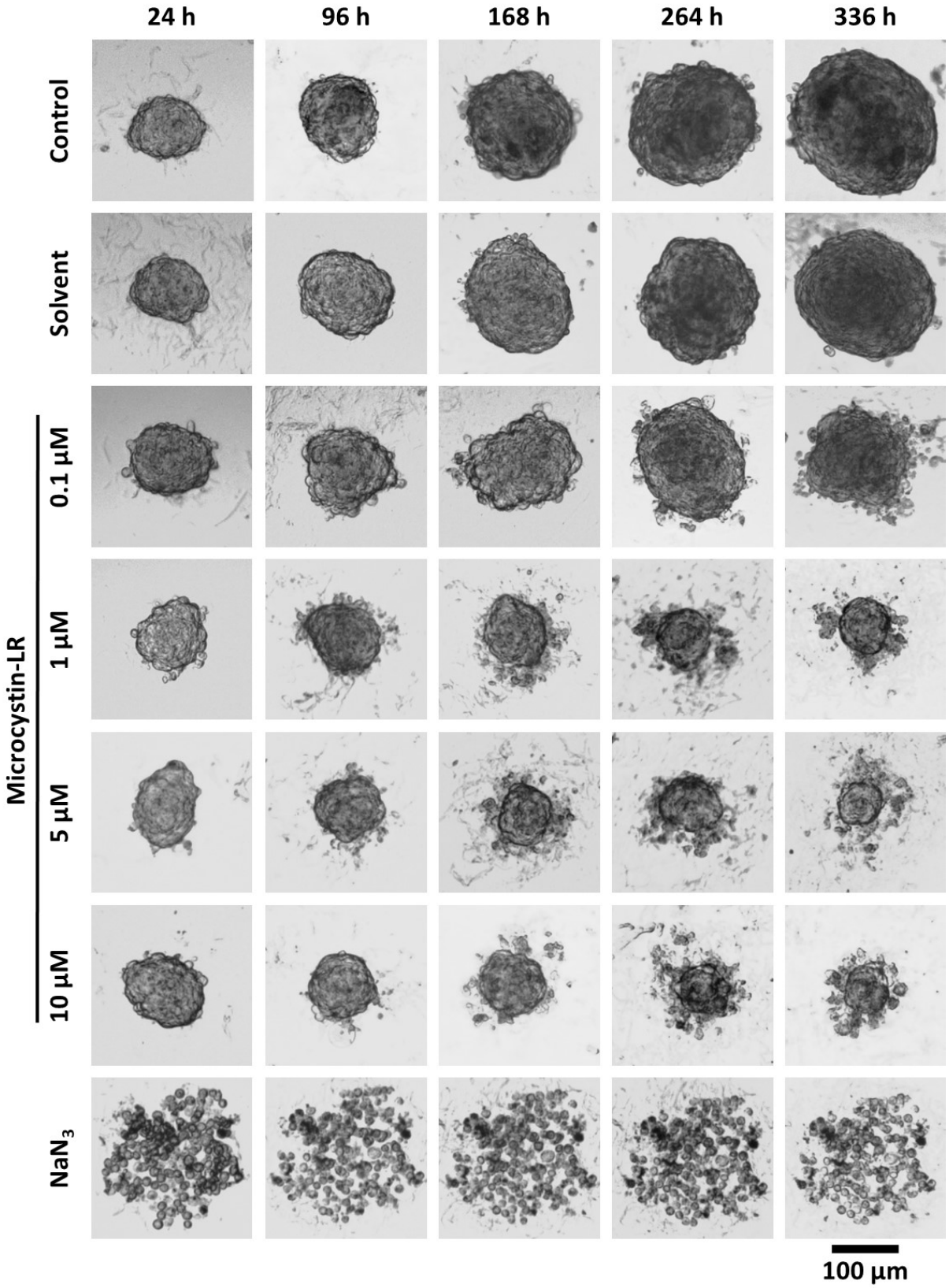
Supporting Figure S2. Effects of microcystin-LR (MC-LR) on adult human liver stem cells in the monolayer experiment. HL1-hT1 cells in a 96-well plate were treated 48 h post-seeding with MC-LR. Brightfield microphotographs were acquired after 96 h exposure using a 10X objective. Solvent – solvent control (0.2% MeOH, v/v).



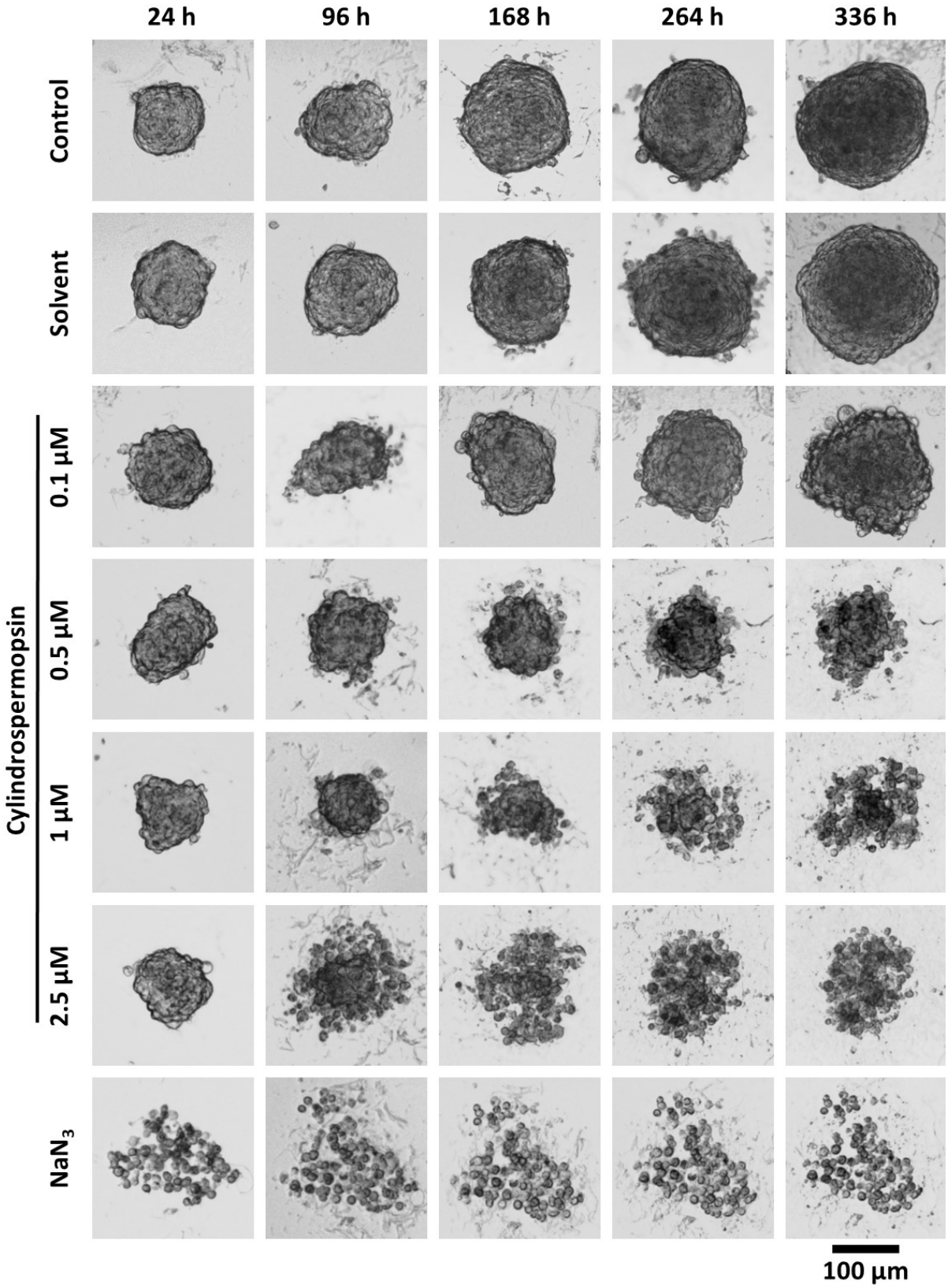
Supporting Figure S3. Effects of cylindrospermopsin (CYN) on adult human liver stem cells in the monolayer experiment. HL1-hT1 cells in a 96-well plate were treated 48 h post-seeding with CYN. Brightfield microphotographs were acquired after 96 h exposure using a 10X objective. Solvent - solvent control (0.2% MeOH, v/v).



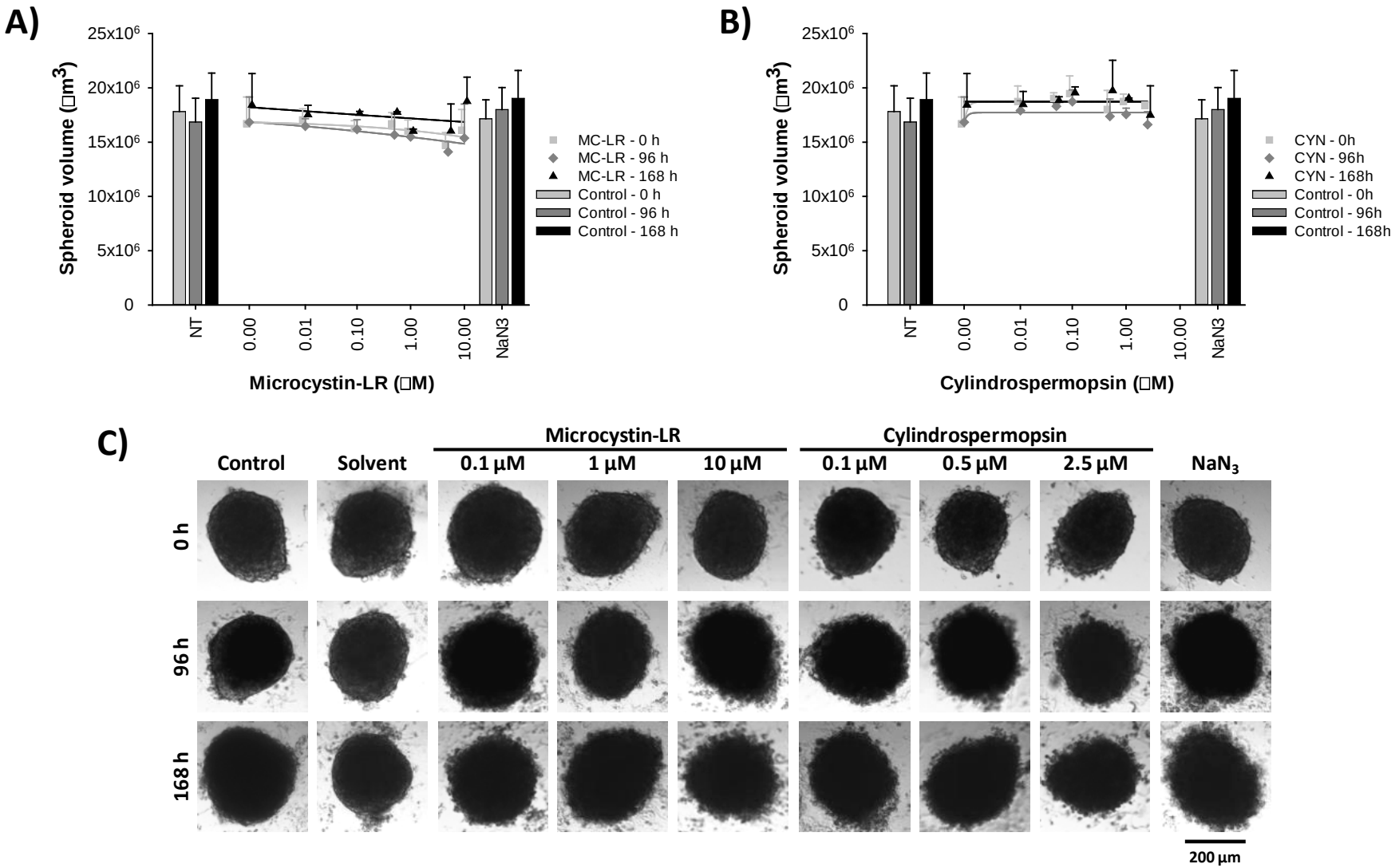
Supporting Figure S4. Effects of microcystin-LR (MC-LR) and cylindrospermopsin (CYN) on the growth of small growing spheroids of adult human liver stem cells. HL1-hT1 cells were seeded into micromolded agarose gel at the initial density 250 cell/spheroid, immediately treated by (A) MC-LR or (B) CYN, and exposed for 336 h. Spheroids were documented periodically by brightfield microscopy (8X magnification) and their size was expressed as spheroid volume. Control - nontreated control, Solvent - solvent control (0.2% MeOH, v/v). Data represent average±SD values derived from independently repeated experiments, the curves in graphs depict a fit of 3-parameter sigmoid function. *Data points below the dashed line were significantly lower (Mann-Whitney test, $p<0.05$) than the solvent control.



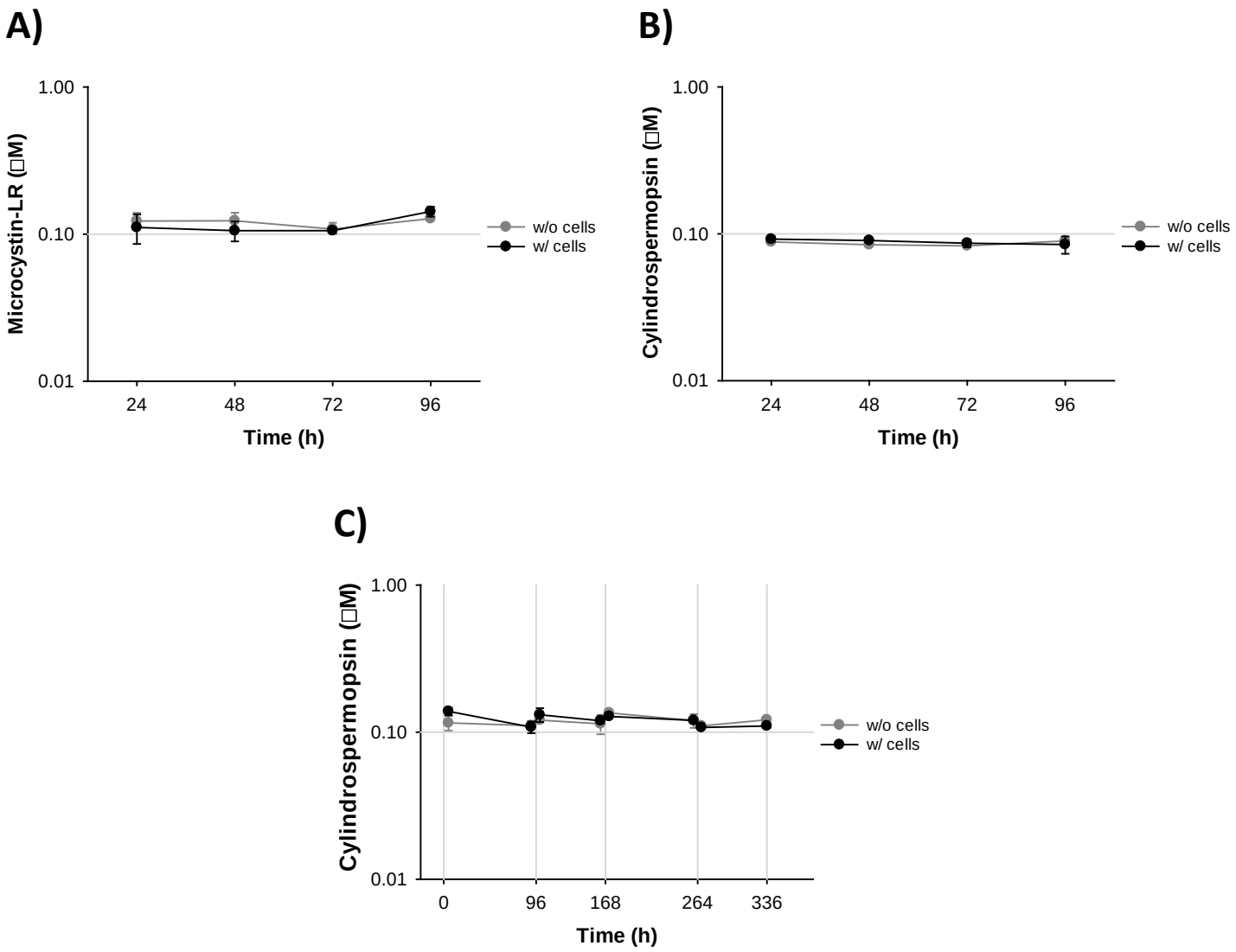
Supporting Figure S5. Effects of microcystin-LR (MC-LR) on the formation and growth of small growing spheroids of adult human liver stem cells. HL1-hT1 cells were seeded into micromolded agarose gel at the initial density 250 cell/spheroid, immediately treated by cyanotoxins MC-LR and exposed for 336 h. Spheroids were documented periodically by brightfield microscopy (8X magnification) and representative spheroid images are shown. Control - nontreated control, Solvent - solvent control (0.2% MeOH, v/v), NaN_3 - positive control (1% sodium azide, w/v).



Supporting Figure S6. Effects of cylindrospermopsin (CYN) on the formation and growth of small growing spheroids of adult human liver stem cells. HL1-hT1 cells were seeded into micromolded agarose gel at the initial density 250 cell/spheroid, immediately treated by cyanotoxins CYN and exposed for 336 h. Spheroids were documented periodically by brightfield microscopy (8X magnification) and representative spheroid images are shown. Control - nontreated control, Solvent - solvent control (0.2% MeOH, v/v), NaN_3 - positive control (1% sodium azide, w/v).



Supporting Figure S7. Effects of microcystin-LR (MC-LR) and cylindrospermopsin (CYN) on the size and morphology of large mature spheroids of adult human liver stem cells. HL1-hT1 cells were seeded into micromolded agarose gels at the initial density 4000 cell/spheroid. Spheroids were incubated for 168 h and then treated with MC-LR or CYN. Spheroids were documented periodically by brightfield microscopy (8X magnification) and their size was expressed as a spheroid volume (A, B). Representative spheroid images (C) are shown for 0 h, 96 h and 168 h of exposure. NT - nontreated control (Control), Solvent - solvent control (0.2% MeOH, v/v), NaN_3 - positive control (1% sodium azide, w/v). Data represent average±SD values derived from independently repeated experiments, the curves in the graphs depict a fit of 3-parameter sigmoid function. Data points below the dashed line were significantly lower (Mann-Whitney test, $p<0.05$) than the solvent control.



Supporting Figure S8. Analysis of microcystin-LR (MC-LR) and cylindrospermopsin (CYN) concentration in the cell culture medium in monolayer and spheroid experiments. In the monolayer experiments, (A) MC-LR or (B) CYN were added into the culture of HL1-hT1 cells at the nominal concentration 0.1 µM, and their concentrations were determined after 24-96 h of exposure. (C) In the spheroid experiments, CYN was added to the cultures of mature spheroids (4000 cell/spheroid) at the initial concentration 0.15 µM in order to reach nominal concentration 0.1 µM after equilibration with the agarose gel. Cyanotoxin concentrations were determined 1 h after the cyanotoxin addition or medium exchange (t=1 h, 97 h, 169 h, 265 h), and prior the medium exchange or experiment termination (t=96 h, 168 h, 264 h, 336 h). Black lines represent cyanotoxin concentrations in the experimental wells with the cells (w/ cells), gray lines represent the control wells without the cells (w/o cells). Exposure start/end and medium exchanges in the spheroid experiments are indicated by the vertical lines, the nominal concentrations of cyanotoxins are indicated by the horizontal lines. Data represent average±SD from independent experiments.

Supporting Table S1. Summary of microcystin-LR (MC-LR) and cylindrospermopsin (CYN) effects on HL1-hT1 cells in monolayer and spheroid experiments – NOEC, LOEC, EC_{25} and EC_{50} values [a]

Cyano-toxin	Exposure Time (h)	Monolayer cultures[b]															
		NRU				Alamar Blue				CFDA-AM				%DCI			
		NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)	NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)	NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)	NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)
MC-LR	48	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
	72	*n.a.* [d]	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	>10,000	>10,000	>10,000	>10,000
	96	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
	120	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	>10,000	>10,000	>10,000	>10,000
CYN	48	100	500	256	726	100	500	381	1,317	1,000	2,500	2,855	5,506	1,000	2,500	880	1,548
	72	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	500	1,000	538	852
	96	100	500	370	753	100	500	261	614	1,000	2,500	2,239	2,910	100	500	401	616
	120	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	100	500	265	454

Cyano-toxin	Exposure Time (h)	Spheroid cultures[c]															
		Small growing spheroids (250 cell/spheroid)								Large mature spheroids (4000 cell/spheroid)							
		Relative Spheroid Growth				Alamar Blue				Relative Spheroid Growth				Alamar Blue			
		NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)	NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)	NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)	NOEC (nM)	LOEC (nM)	EC25 (nM)	EC50 (nM)
MC-LR	96	1,000	5,000	888	2,145	*n.a.*	*n.a.*	*n.a.*	*n.a.*	>10,000	>10,000	>10,000	>10,000	<10	10	11	43
	168	<100	100	68	390	*n.a.*	*n.a.*	*n.a.*	*n.a.*	>10,000	>10,000	>10,000	>10,000	<10	10	6	79
	264	<100	100	33	202	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
	336	<100	100	29	162	<100	100	47	146	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CYN	96	100	500	153	377	*n.a.*	*n.a.*	*n.a.*	*n.a.*	>2,500	>2,500	>2,500	>2,500	<10	10	3	104
	168	<100	100	28	145	*n.a.*	*n.a.*	*n.a.*	*n.a.*	>2,500	>2,500	>2,500	>2,500	<10	10	7	54
	264	<100	100	33	145	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
	336	<100	100	36	124	<100	100	23	119	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*

[a] EC_{25} / EC_{50} (effective concentrations inducing 25% or 50% inhibition of growth or viability) values were estimated using a 3-parameter sigmoid function; NOEC (No Observed Effect Concentration) / LOEC (Lowest Observed Effect Concentration) values were assessed by Mann-Whitney test ($p<0.05$)

[b] In monolayer experiments, cell growth and viability were evaluated using Neutral Red Uptake (NRU), Alamar Blue, and CFDA-AM assays as well as using impedimetric analysis (%DCI – control-normalized Delta Cell Index)

[c] In spheroid experiments, the values were calculated from control-normalized spheroid growth (Relative Spheroid Growth) and from viability assay (Alamar Blue).

[d] n.a. – not available, the condition was not tested