

Supplementary Material and Methods

Development of CRCPM-derived PDO

PDOs were seeded in 24-multi well plates and cultured using the 6 different media combinations. The impact of these six media on PDOs growth was determined by counting the number of PDOs present in each well (Supplementary Fig. S2, Supplementary Table S1) using Qupath software (<https://qupath.github.io>, version 0.2.3). Images used for Qpath analyses were acquired with Aperio Leica ScanScope XT (Leica Biosystems, Wetzlar, Germany). Three different fields were counted for each experiment.

The specific composition of each medium was the following:

- Medium #1: DMEM-F12; B27; Glutamax
- Medium #2: DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I
- Medium #3: DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01
- Medium #4: DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; EGF; A83-01; Noggin
- Medium #5: DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01; SB202190; Noggin
- Medium #6: DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01; Noggin

Nucleic acids extraction and Mutational analysis

DNA was extracted from PDOs (PM1, PM2, PM3, PM4, PM5, PM6 and PM7) and from formalin-fixed paraffin-embedded (FFPE) sections of the tumors from which they were derived using the DNeasy Blood&Tissue kit (QIAGEN, Germany) and the Masterpure Complete DNA Purification Kit (Lucigen-Biosearch Technologies, Middleton, WI, USA) respectively. DNA was quantified with Nanodrop™ 1000 (ThermoFisher Scientific, USA) and Qubit 2.0 fluorimeter (Thermofisher Scientific, USA) and used for mutational analysis with the 50-gene Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies, USA). The mutational status of a group genes was assessed using targeted next-generation sequencing (T-NGS), with the Ion-Torrent™ Personal Genome Machine platform (Life Technologies, USA, genes analyzed: *ABL1*, *AKT*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNAI1*, *GNAQ*, *GNAS*, *HNFI1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR* (*VEGFR2*), *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RBI*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53* and *VHL*).

The Ion AmpliSeq Library Kit 2.0 (Life Technologies) was used to amplify 40 ng of DNA according to the manufacturer's instructions (MAN0006735 rev 5.0). Libraries were purified using AMPure Beads XP (Beckman Coulter) and PCR-amplified for a total of five cycles. Emulsion PCR and sample enrichment were completed using the IonOne Touch 2 instrument. Sequencing was done on Ion Torrent PGM using Ion 316 Chips and the Ion-PGM 200 sequencing kit (Life Technologies), according to the manufacturer's instructions.

PGM sequencing data were initially processed using the Ion Torrent platform-specific Torrent Suite software to generate sequence readouts, align them on the reference genome Hg19, trim the adapter sequences, filter and discard any poor signal-profile readouts. Variant calling from the sequencing data was done with the Variant Caller plug-in. The filtered variants were examined visually using the Integrative Genomic Viewer tool to check their quality level and confirm the variant's presence on both the "+" and the "-" strand. The resulting variants were then recorded using the Ensemble Variant Effect Predictor pipeline, COSMIC database, dbSNP database and MyCancerGenome database (<http://www.mycancergenome.org/>).

Supplementary Material and Methods

Immunohistochemistry analyses

FFPE blocks were prepared for immunohistochemistry (IHC) as previously described [1]. PDOs were included in paraffin and cut into slices (3 μm); paraffin was removed with xylene and slices were rehydrated using decreasing concentrations of ethanol, and washed with water. IHC was performed with the following antibodies: CK AE1/AE3, CK20, CDX2 and ki-67, following the specifications reported in Supplementary Table S6. Images were acquired with a DM6000B microscope (Leica). Antigen retrieval was carried out using preheated target retrieval solution (pH 6.0) for 30 minutes. Tissue sections were blocked with FBS serum in PBS for 60 min and incubated overnight with primary antibody. The antibody binding was detected using a polymer detection kit (GAM/GAR-HRP, Microtech, Italy) followed by a diaminobenzidine chromogen reaction (Peroxidase substrate kit, DAB, SK-4100; Vector Lab, USA). All sections were counterstained with Mayer's hematoxylin.

QuPath analyses

Cell counting and percentage estimation of cCASPASE3-positive cells were performed using Qupath software (<https://qupath.github.io>, version 0.2.3). The percentage of positive cells was calculated by dividing the number of positive cells present in each field by the total number of cells (cCASPASE⁺ + cCASPASE3⁻) in the same field.

CellTiterGlo® 3D Cell Viability Assay and Caspase-Glo® 3 Assay protocol

PDOs and co-culture viability and CASPASE 3 activation were assessed using a CellTiterGlo® 3D Cell Viability Assay kit and a Caspase-Glo® 3 assay, respectively (Promega, USA) on a TECAN spark microplate reader (Tecan Trading AG, Switzerland) following the manufacturers' instructions. Data were normalized to the mean of the untreated group (UNT: PDO treated with DMEM-F12 only). All the experiments were performed in triplicate.

Immunofluorescence analyses on co-culture models

Immunofluorescence analyses on co-cultures were performed as previously reported [2-3]. CAFs were seeded in an 8-wells chambered coverslip (Ibidi) as described above. Co-cultures were directly fixed in plate with 4 % paraformaldehyde (Sigma-Aldrich) for 15 min at 4 °C, then the plate was swirled and the co-cultures were incubated in 4 % paraformaldehyde for other 15 min at 4 °C. Co-cultures were permeabilized with 0.5 % Triton X-100 (Sigma-Aldrich) for 30 min at RT on a rotor (100 rpm) and blocked for one hour with 5 % BSA and 0.1 % Triton X-100 in PBS 1X at RT on a rotor (100 rpm). Primary antibody mouse anti α -Smooth Muscle Actin (α -SMA) conjugated with Alexaflour 488 dye (1:500, Cell Signalling Technology, clone 1A4, #46489) and rabbit anti anti-pan cytokeratin (1:100, Abcam, clone C-11, ab7753) were used overnight at 4 °C and detected using secondary goat anti rabbit AlexaFluor 568 (1:500, Thermo Fisher, A11036) for one hour at RT. Nuclei were stained with DAPI (1:25000, Thermo Fisher, D1306). Images were acquired on DM600B microscope (Leica, Germany) equipped with a 100 W mercury lamp and analyzed using Cytovision software (Leica, Germany).

Immunoblotting: Cancer –associated fibroblast characterization

CAF were lysed and their protein content was extracted as in [1]. For each sample, 40 μg of protein extract were separated on 4–12% polyacrylamide gels and incubated with the following primary antibodies: α -SMA; FAP; E-Cadherin and Vinculin. The signals were detected using enhanced chemiluminescence, and protein levels were quantified using Image Lab Software (Bio-Rad, Hercules, CA, USA) (Supplementary Table S3).

References

1. Varinelli L, Guaglio M, Brich S, Zanutto S, Belfiore A, Zanardi F, et al. Decellularized Normal and Tumor Extracellular Matrix as Scaffold for Cancer Organoid Cultures of Colorectal Peritoneal Metastases. *J Moll Cell Biol.* 2023;6;14(11):mjac064.
2. Bergdorf KN, Phifer CJ, Bechard ME, Lee MA, McDonald OG, Lee E, et al. Immunofluorescent staining of cancer spheroids and fine-needle aspiration-derived organoids. *STAR Protoc.* 2021;18,2(2):100578.
3. Kodba S and Chaigne A. A quick, cheap, and reliable protocol for immunofluorescence of pluripotent and differentiating mouse embryonic stem cells in 2D and 3D colonies. *STAR Protoc.* 2023;17;4(1):102000.

Supplementary informations

Supplementary Table S1

Factor	Description	Vendor	Working Concentration
Gentamicin	Antibiotic	ThermoFisher Scientific	50 ng/ml
HEPES	Buffer	ThermoFisher Scientific	10 mM
L-Glutamine (GlutaMAX)	Cell culture supplement	ThermoFisher Scientific	2 mM
B27	Cell culture supplement	ThermoFisher Scientific	1:50
Gastrin-1, recombinant human	Recombinant protein	Sigma Aldrich	10 nM
N-acetylcysteine	Colonic niche factor	Wako	1 mM
EGF, recombinant human	Recombinant protein	ThermoFisher Scientific	50 ng/ml
Noggin, recombinant human	Recombinant protein	Preprotech	100 ng/ml
Prostaglandin E2	Colonic niche factor	Tocris	100 nM
A83-01	p38 inhibitor	Tocris	500 nM
SB202190	ROCK inhibitor	Sigma Aldrich	10 µM

Supplementary Table S1: The complete list of growth factors and media supplements, with their working concentrations used for TDOs culturing.

Supplementary information

Supplementary Table S2

PDO culture	Medium composition
C1	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I
C2	DMEM-F12; B27; Glutamax
C3	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01
C4	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; EGF; A83-01; Noggin
C6	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01; Noggin
PM1	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I
PM2	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I
PM3	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01; SB202190; Noggin
PM4	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01; SB202190; Noggin
PM5	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I
PM6	DMEM-F12; B27; Glutamax
PM7	DMEM-F12; B27; Glutamax; N-acetylcysteine; prostaglandin-E2; gastrin-I; A83-01; SB202190; Noggin

Supplementary Table S2: The specific media formulation for C1, C2, C3, C4, C5, C6 PM1, PM2, PM3, PM4, PM5, PM6 and PM7 PDO cultures.

Supplementary Table S3

Antigen (human)	Host	Vendor	Dilution	Incubation
α -SMA	Mouse	Cell Signaling Technology	1:1000	O/N at 4 °C
FAP	Rabbit	Cell Signaling Technology	1:1000	O/N at 4 °C
E-Cadherin	Mouse	Cell Signaling Technology	1:1000	O/N at 4 °C
Vinculin	Mouse	Cell Signaling Technology	1:1000	O/N at 4 °C

Supplementary Table S3: The primary antibodies and the experimental conditions used for WB analyses of CAFs.

Supplementary table S4: Determination of the clinically relevant doses

Employed Drugs	HIPEC Clinical Scheme	Normalized Concentration (mg/L)*	mol (g/MW)	Clinically relevant dose [M]**
1 (MMC)	35 mg/m ² MMC in 2.5 L/m ² perfusate	35mg/m ² / 2.5L/m ² = 14mg/L	0.014 g/334.33 g/mol = 4.19 x 10 ⁻⁵ mol	4.19 x 10 ⁻⁵ mol / 1L = 41.9 μM
2 (MMC + CDDP)	3.5mg/m ² /L MMC + 25 mg/m ² /L CDDP in perfusate	3.5mg/m ² / 1L/m ² = 3.5mg/L MMC 25mg/m ² / 1L/m ² = 25mg/L CDDP	0.0035g / 334.33g/mol = 1.04 x 10 ⁻⁵ mol (MMC) 0.025g / 301.11g/mol = 8.30 x 10 ⁻⁵ mol (CDDP)	1.04 x 10 ⁻⁵ / 1L = 10.4 μM MMC 8.30 x 10 ⁻⁵ mol / 1L = 83 μM CDDP
3 (DOX + CDDP)	15.25 mg/L DOX + 43 mg/L CDDP in perfusate	15.25mg/L / 1L = 15.25mg/L (DOX) 43mg/L / 1L = 43 mg/L (CDDP)	0.015250g / 543.52g/mol = 2.81 x 10 ⁻⁵ mol (DOX) 0.043g / 301.11 g/mol = 1.43 x 10 ⁻⁴ mol (CDDP)	2.81 x 10 ⁻⁵ mol / 5L = 5.61 μM DOX 1.43 x 10 ⁻⁴ mol / 5L = 28.6 μM CDDP
4 (L-OHP _{Low-dose})	200 mg/m ² in 2 L 5 % Glucosate	200mg/m ² / 2L/m ² = 100mg/L	0.100g / 397.28g/mol = 5.03 x 10 ⁻⁴ mol	5.03 x 10 ⁻⁴ mol / 2L = 252 μM
5 (L-OHP _{High-dose})	460 mg/m ² in 2 L 5 % Glucosate	460mg/m ² / 2L/m ² = 230mg/L	0.230g / 397.28g/mol = 1.16 x 10 ⁻³ mol	1.16 x 10 ⁻³ mol / 2L= 579 μM

Supplementary table S4: Determination of the clinically relevant dose for each HIPEC scheme tested. * Concentrations were obtained by normalizing each concentrations using 1 L as the final reference dilution volume. ** Clinically relevant doses were obtained using 1 L (scheme 1 and 2), 5 L (scheme 3) and 2 L (scheme 4 and 5) as the final reference dilution volumes.

Supplementary table S5: NGS analysis (PDOs Vs Tissues)

ID	Mutation in PDO	Mutation in Tissue
C1 [18]	<i>KRAS</i> ; p.G12S <i>TP53</i> ; p.R175H <i>RAD51C</i> ; p.D253H	<i>KRAS</i> ; p.G12S <i>TP53</i> ; p.R175H
C2 [18]	<i>BRAF</i> ; p.V600E <i>TP53</i> ; p.P152L <i>BARD</i> ; p.A40V	<i>BRAF</i> ; p.V600E <i>TP53</i> ; p.P152L
C3 [18]	<i>KRAS</i> ; p.G12S <i>TP53</i> ; p.R273C <i>FGR1</i> ; amp	<i>KRAS</i> ; p.G12S <i>TP53</i> ; p.R273C <i>FGR1</i> ; amp
C4 [18]	<i>KRAS</i> ; p.G12S <i>BARD</i> ; p.V507M <i>MSH3</i> ; p.A55A60del <i>MSH6</i> ; p.R62R	<i>KRAS</i> ; p.G12S
C6 [18]	<i>KRAS</i> ; p.G12S <i>TP53</i> ; p.G245V <i>APC</i> ; p.Q1406Fs <i>SMAD4</i> ; pD315H	<i>KRAS</i> ; p.G12S <i>TP53</i> ; p.G245V <i>APC</i> ; p.Q1406Fs <i>SMAD4</i> ; pD315H
PM1	<i>APC</i> ; p.E1309KFsTer5 <i>KRAS</i> ; p.G12D <i>TP53</i> ; p.R248W	<i>APC</i> ; p.E1309KFsTer5 <i>KRAS</i> ; p.G12D <i>TP53</i> ; p.R248W
PM2	<i>APC</i> ; p.R1450* <i>KRAS</i> ; p.Q61H <i>TP53</i> ; p.R2486Q	<i>APC</i> ; p.R1450* <i>KRAS</i> ; p.Q61H <i>TP53</i> ; p.R2486Q
PM3	<i>KRAS</i> ; p.G12C	<i>KRAS</i> ; p.G12C
PM4	<i>KRAS</i> ; p.Q61H <i>TP53</i> ; p.R175H	<i>KRAS</i> ; p.Q61H <i>TP53</i> ; p.R175H
PM5	<i>KRAS</i> ; p.G12S	<i>KRAS</i> ; p.G12S
PM6	<i>BRAF</i> ; p.V600E <i>TP53</i> ; p.P152L	<i>BRAF</i> ; p.V600E <i>TP53</i> ; p.P152L <i>SMAD4</i> ; pL146* <i>KDR</i> ; p.R962C
PM7	<i>APC</i> ; p.M1383dup <i>TP53</i> ; p.R248Q	<i>APC</i> ; p.M1383dup <i>TP53</i> ; p.R248Q

Supplementary Table S5: The mutational profile of PDOs and their corresponding tissue.

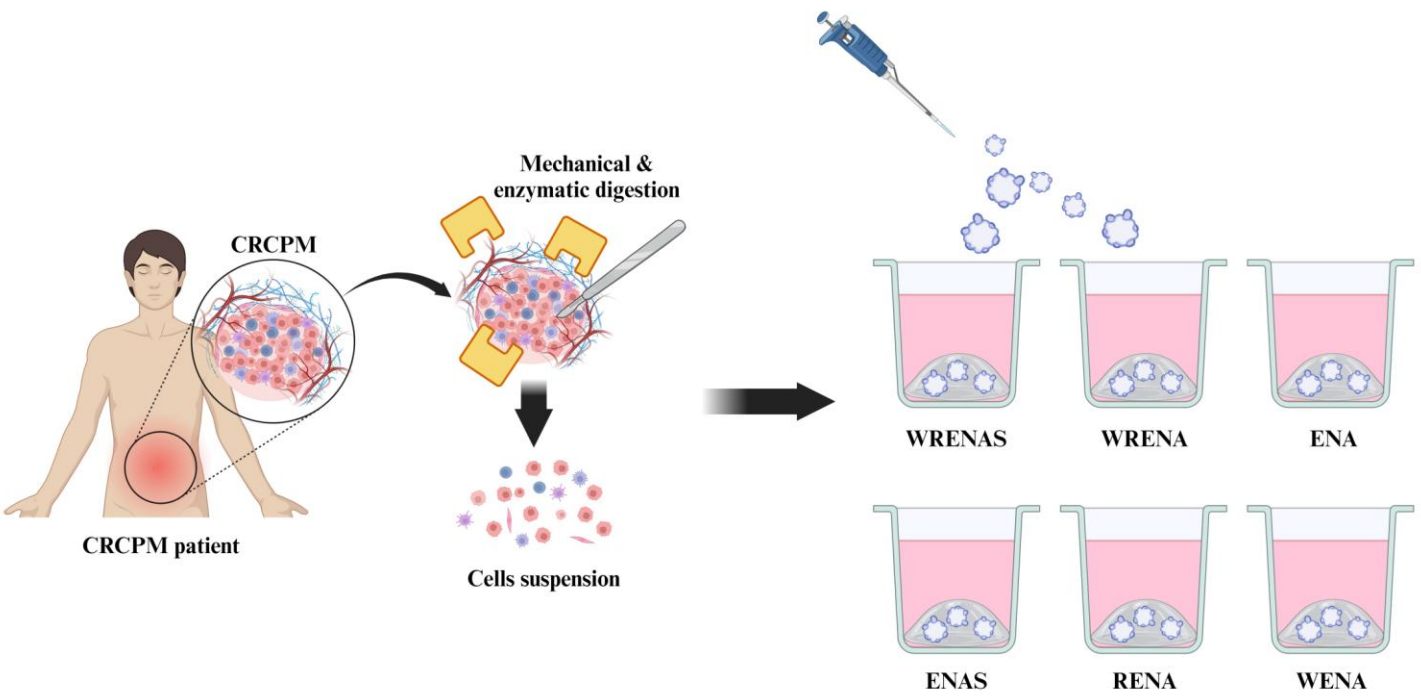
Amp: amplified; del: deleted; dup: duplication; Fs: frameshift mutation; FsTer5: frameshift mutation that generates a stop codon; * stop codon.

Supplementary Table S6

Antigen (human)	Host	Clone	Vendor	Dilution	Antigen retrieval solution
Ki-67	Mouse	MIB-1	Dako	1:400	5 mM EDTA (pH 8), 10 min, 96 °C
CK20	Mouse	Ks20.8	Dako	1:500	5 mM EDTA (pH 8), 30 min, 96°C
CK AE1/AE3	Mouse	AE1+AE3	Dako	1:100	10 mM Citrate (pH 6), 15 min, 96°C
CDX2	Mouse	CDX2_88	Dako	1:50	5 mM EDTA (pH 8), 30 min, 96°C

Supplementary Table S6: Primary antibodies and experimental conditions used for IHC analyses.

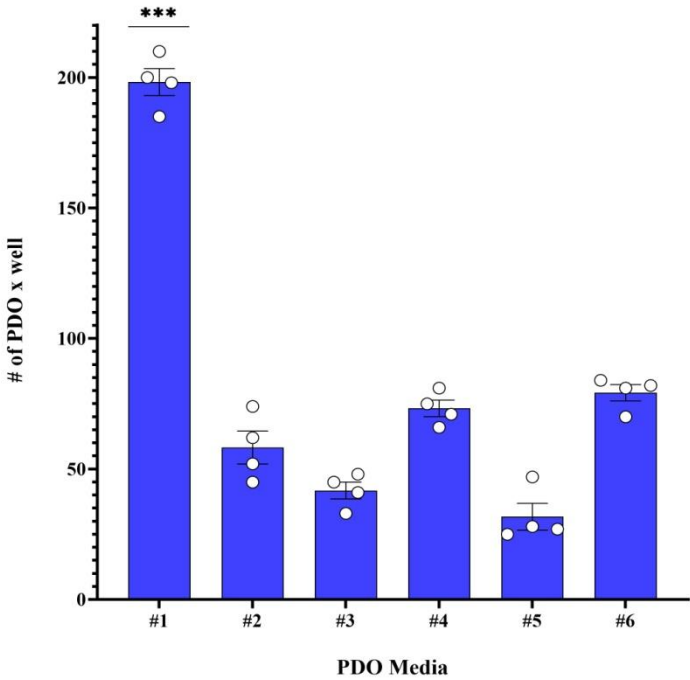
Supplementary Figure S1



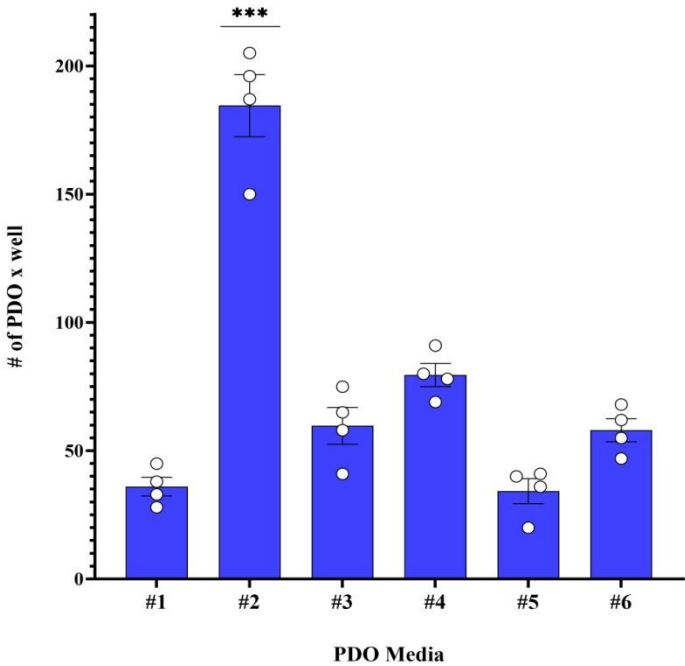
Entry	Entry name	Description
W	Wnt3A	Recombinant protein
R	R-spondin-1	Recombinant protein
E	EGF	Recombinant protein
N	Noggin	Recombinant protein
A	A83-01	Alk inhibitor
S	SB202190	Anti-p38 inhibitor

Supplementary Figure S2

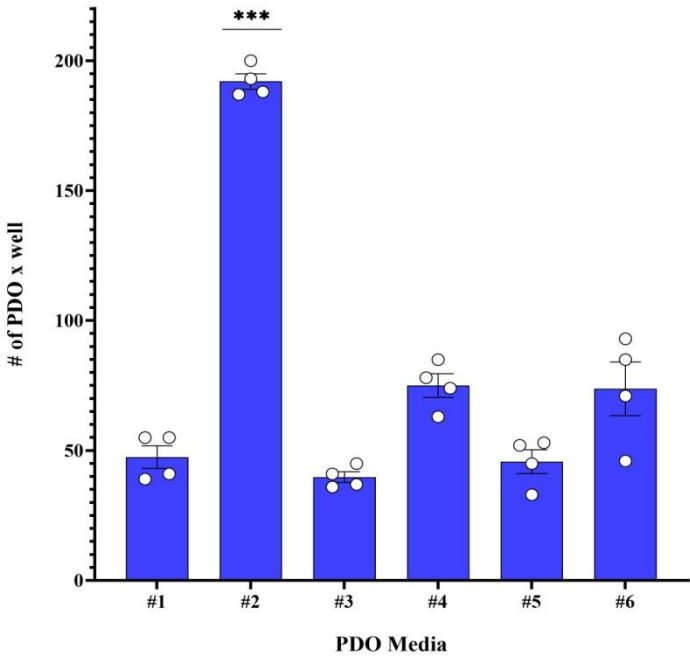
C1 PDO line



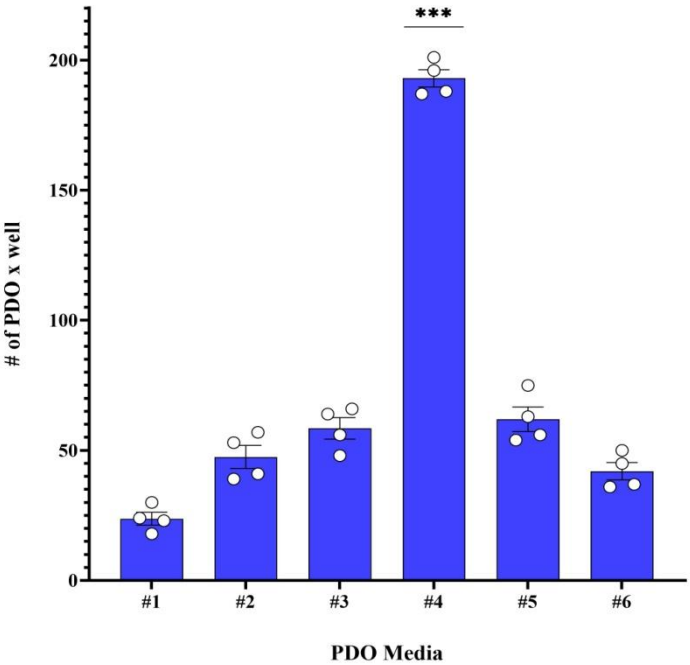
C2 PDO line



C3 PDO line

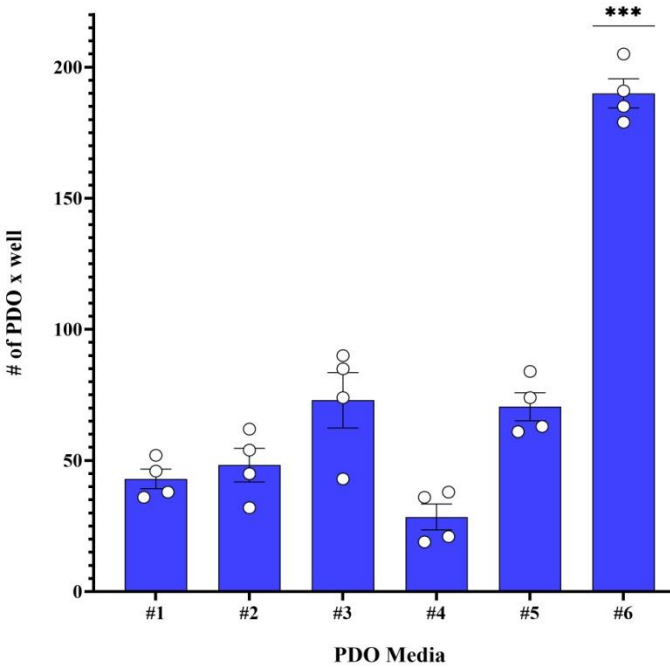


C4 PDO line

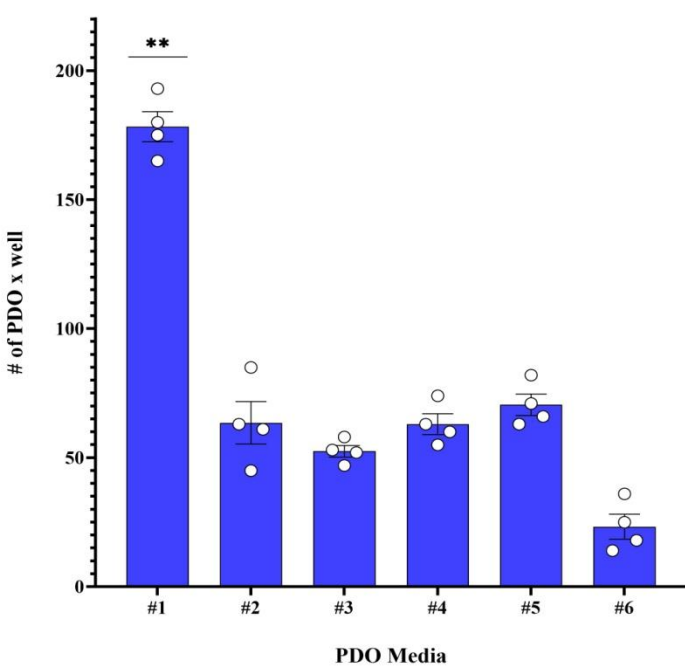


Supplementary Figure S2

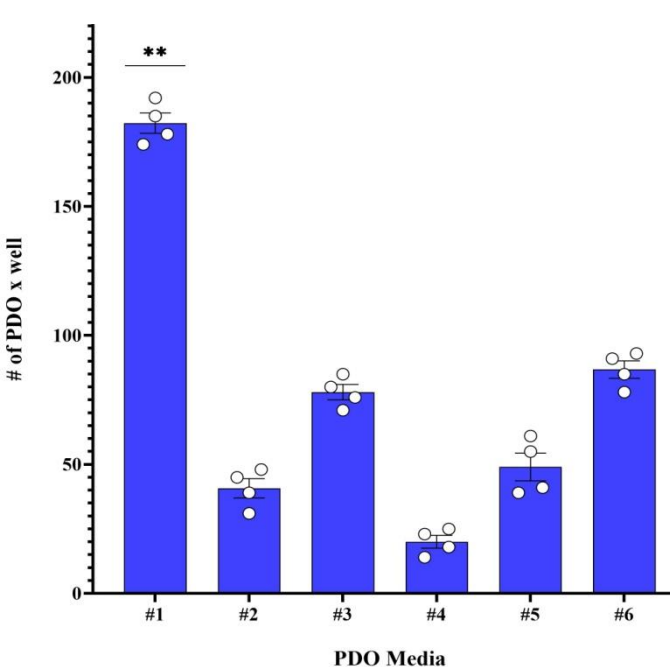
C6 PDO line



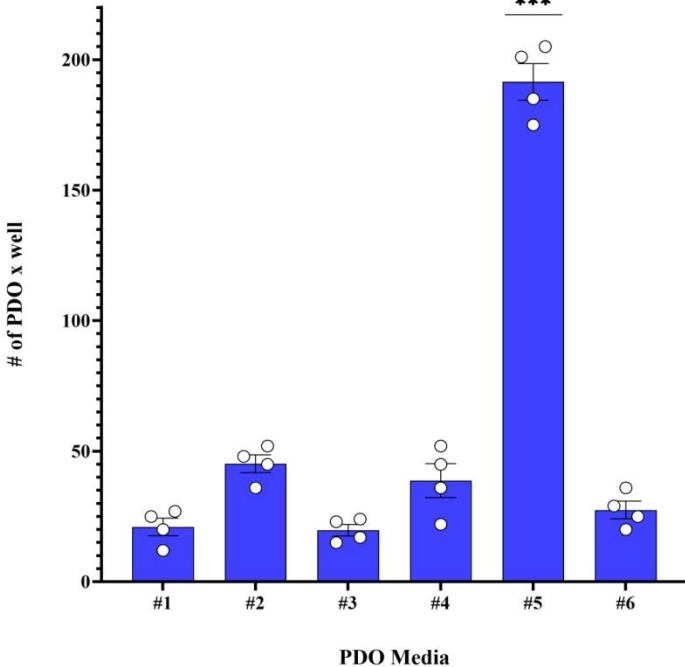
PM1 PDO line



PM2 PDO line

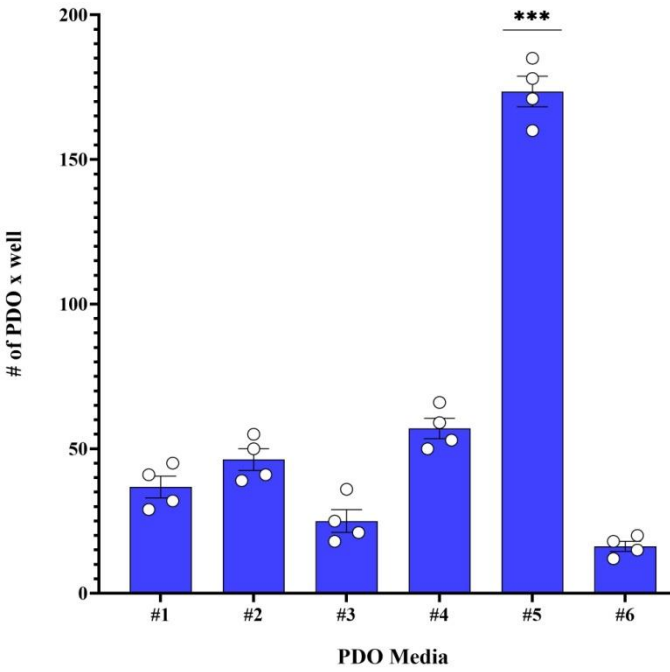


PM3 PDO line

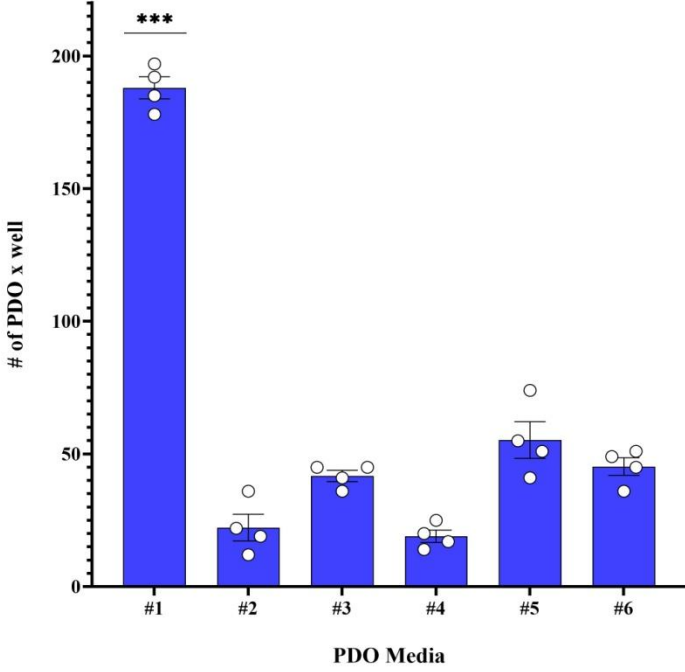


Supplementary Figure S2

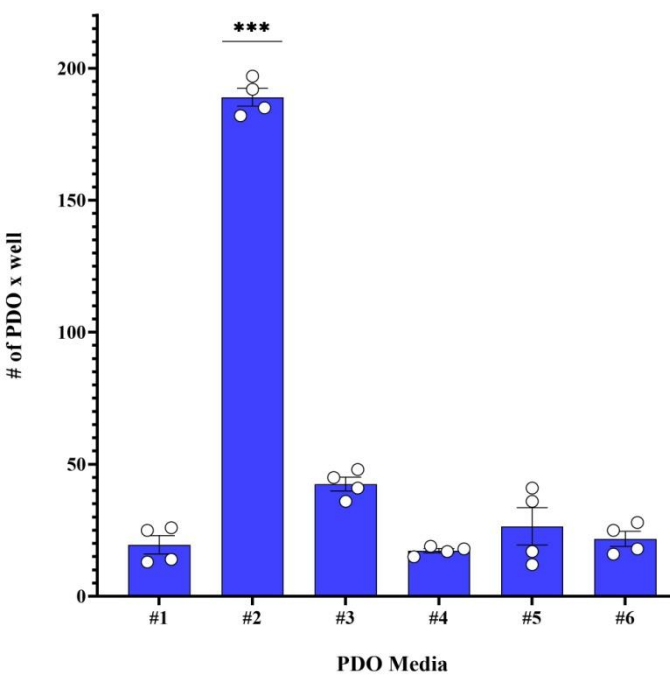
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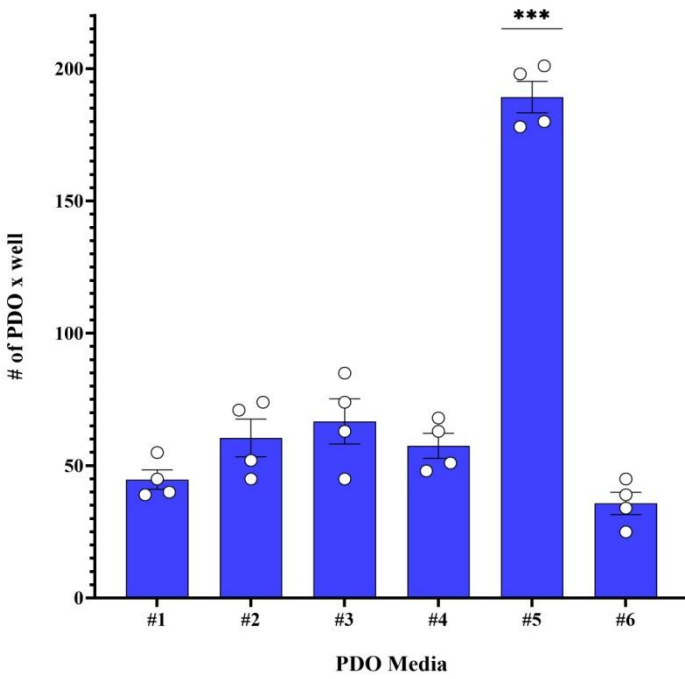
PM5 PDO line



PM6 PDO line

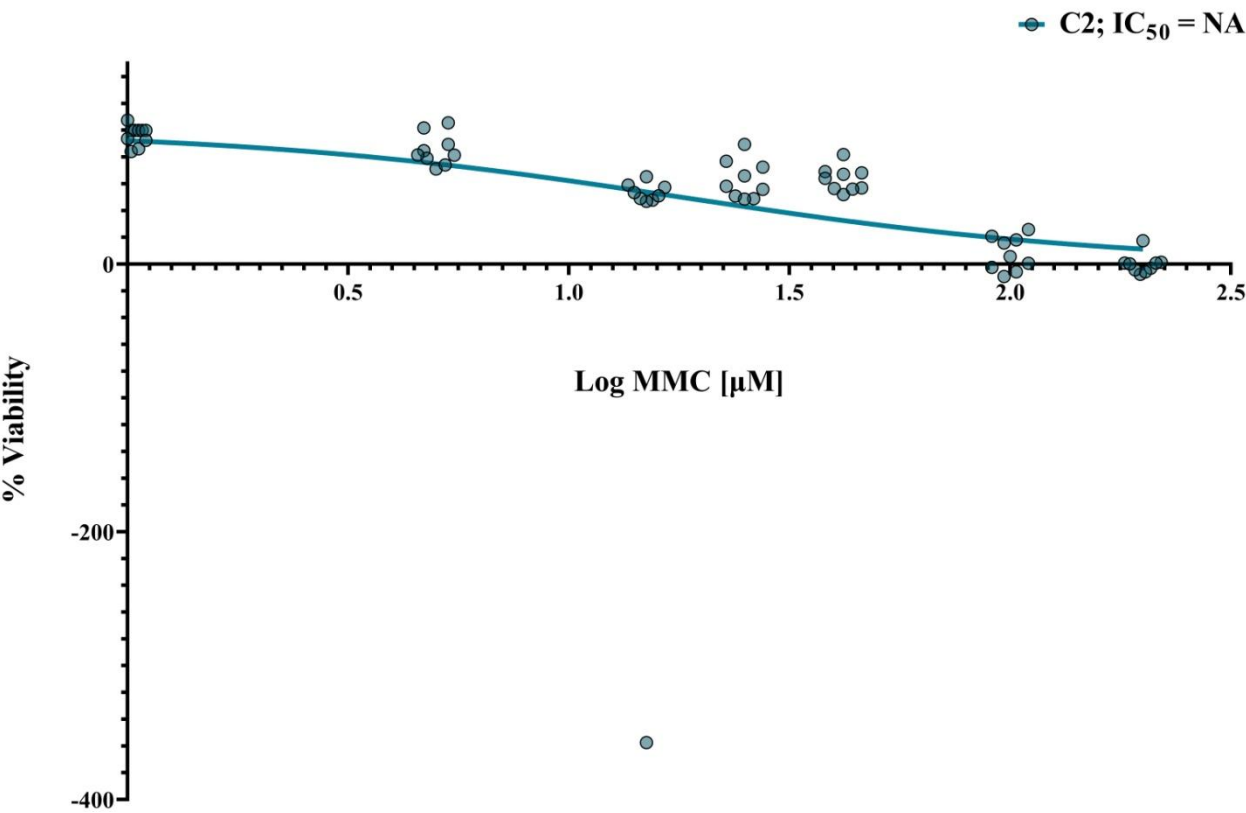


PM7 PDO line

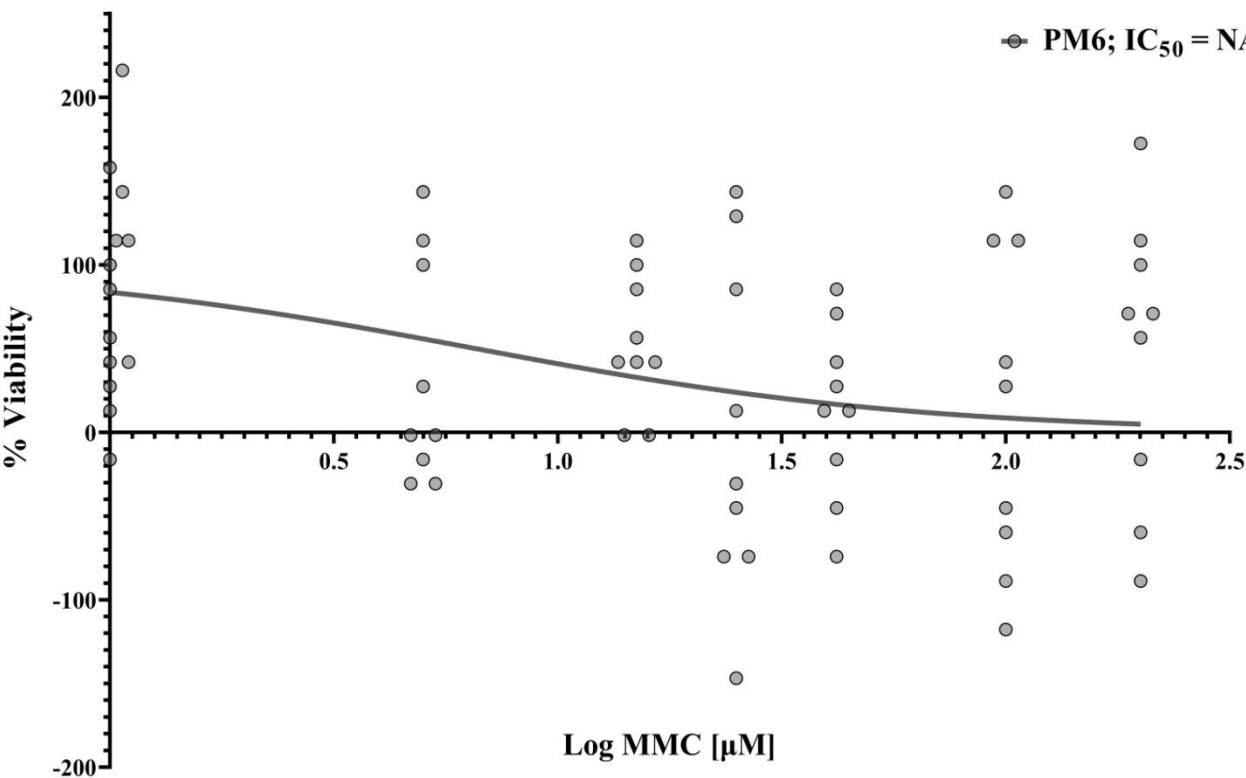


Supplementary Figure S3

HIPEC treatments - Scheme 1 - C2 PDO line

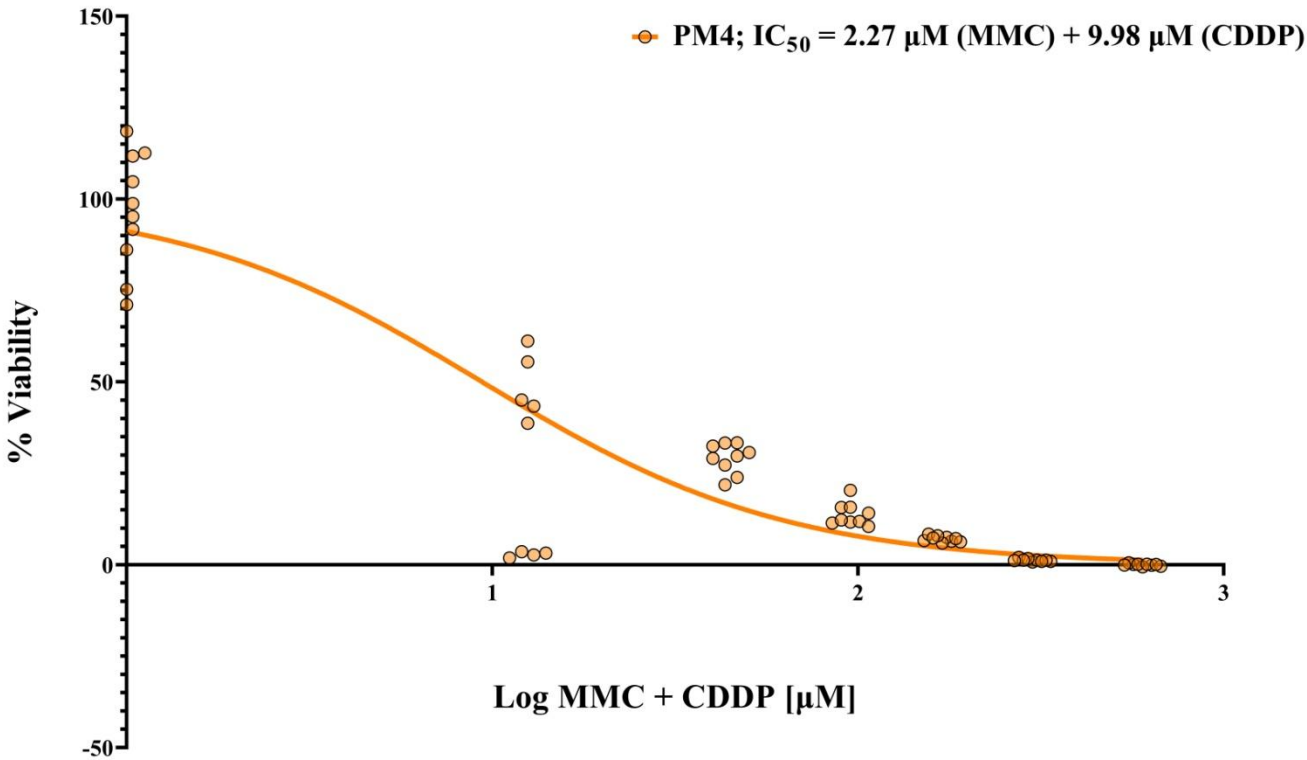


HIPEC treatments - Scheme 1 - PM6 PDO line

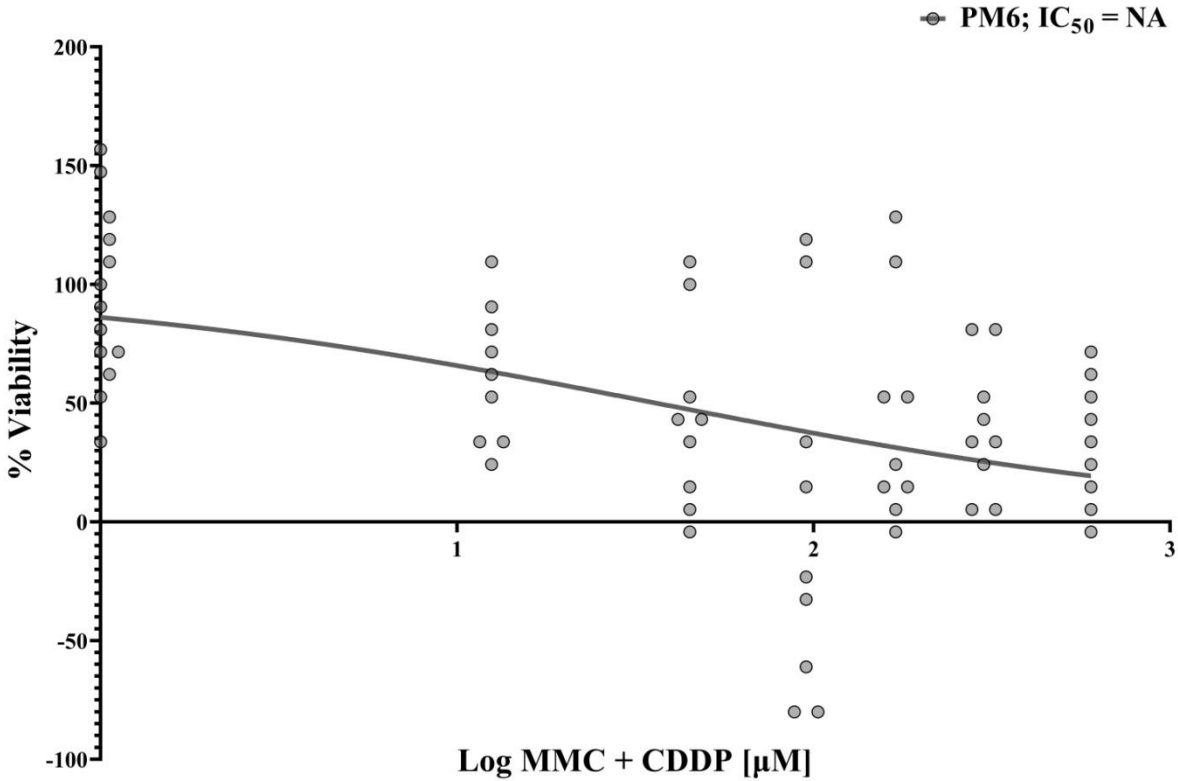


Supplementary Figure S3

HIPEC treatments - Scheme 2 - PM4 PDO line

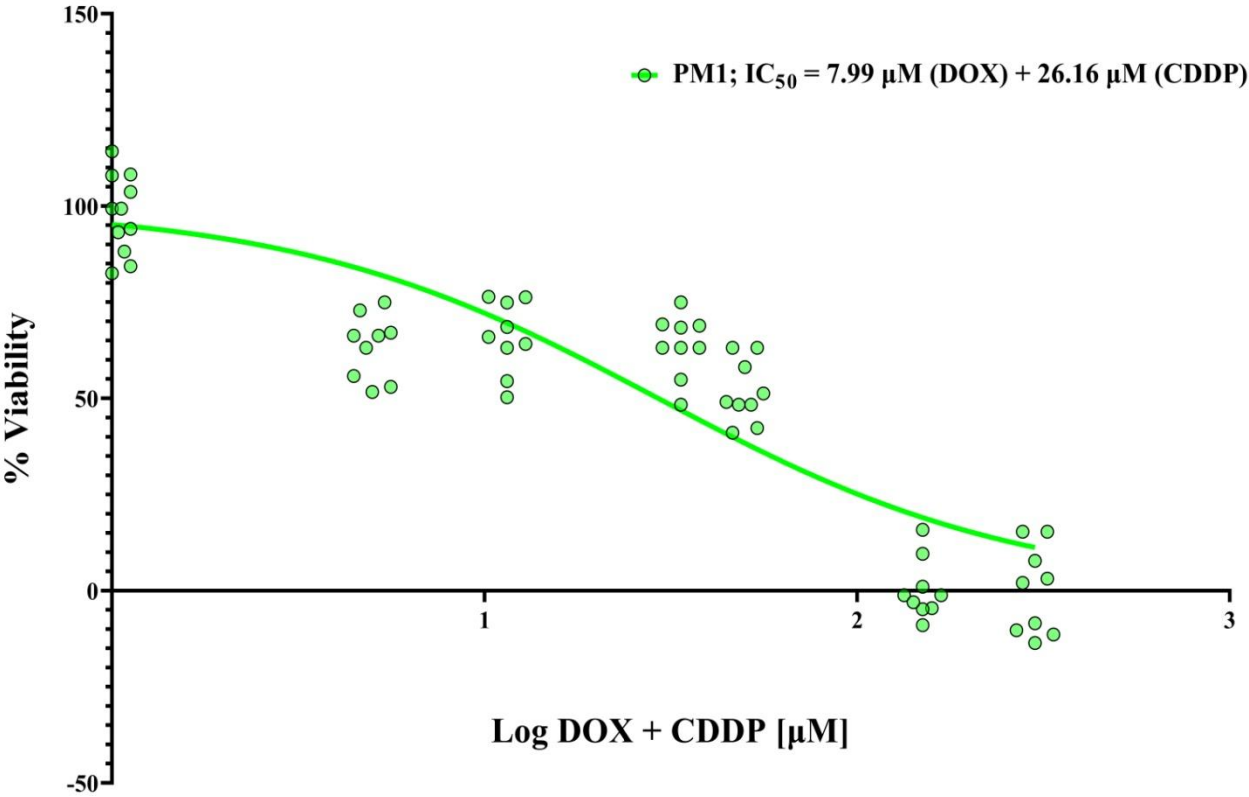


HIPEC treatments - Scheme 2 - PM6 PDO line

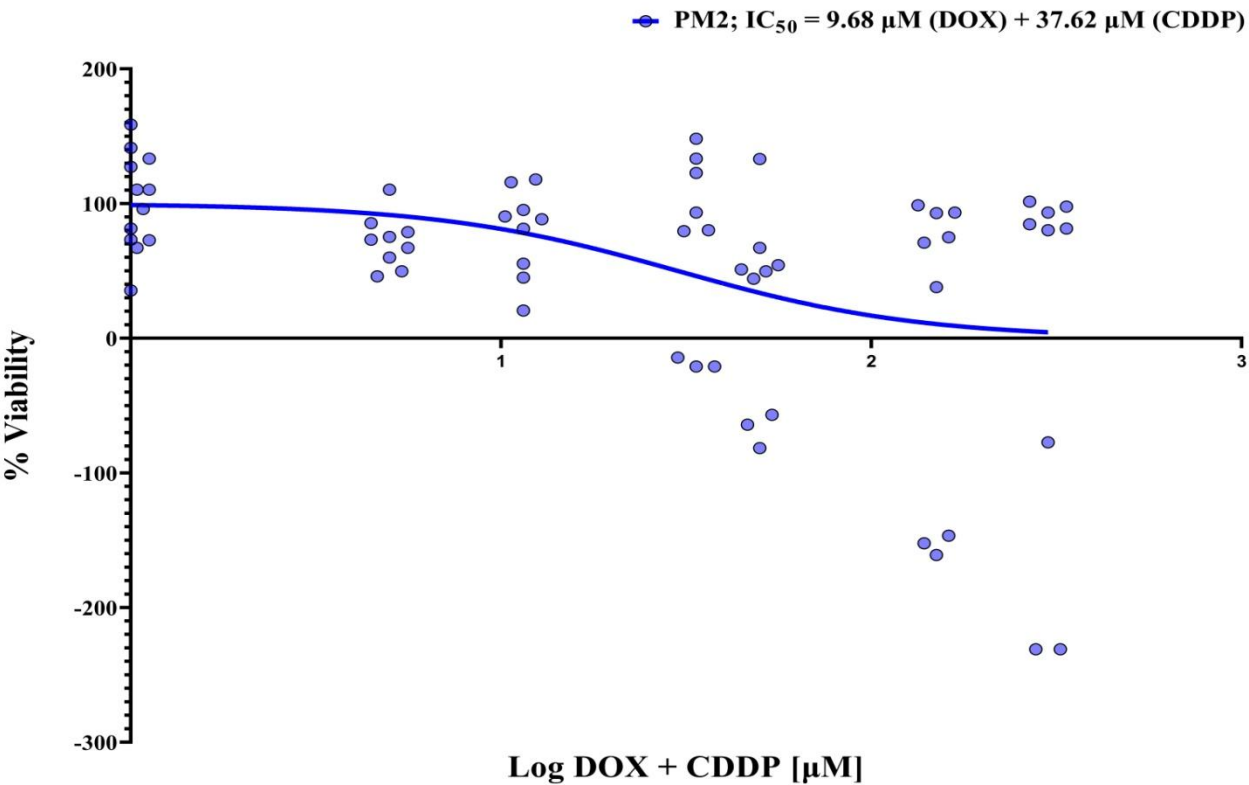


Supplementary Figure S3

HIPEC treatments - Scheme 3 - PM1 PDO line

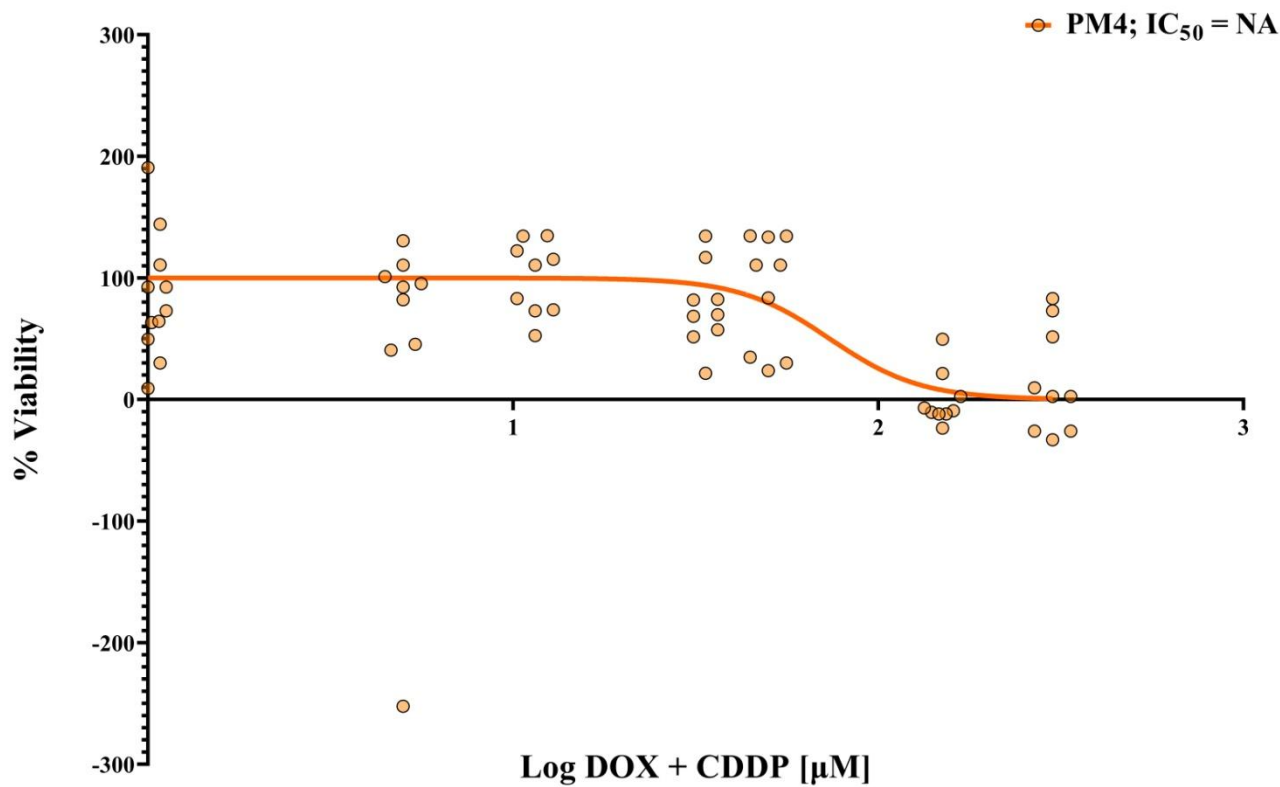


HIPEC treatments - Scheme 3 - PM2 PDO line

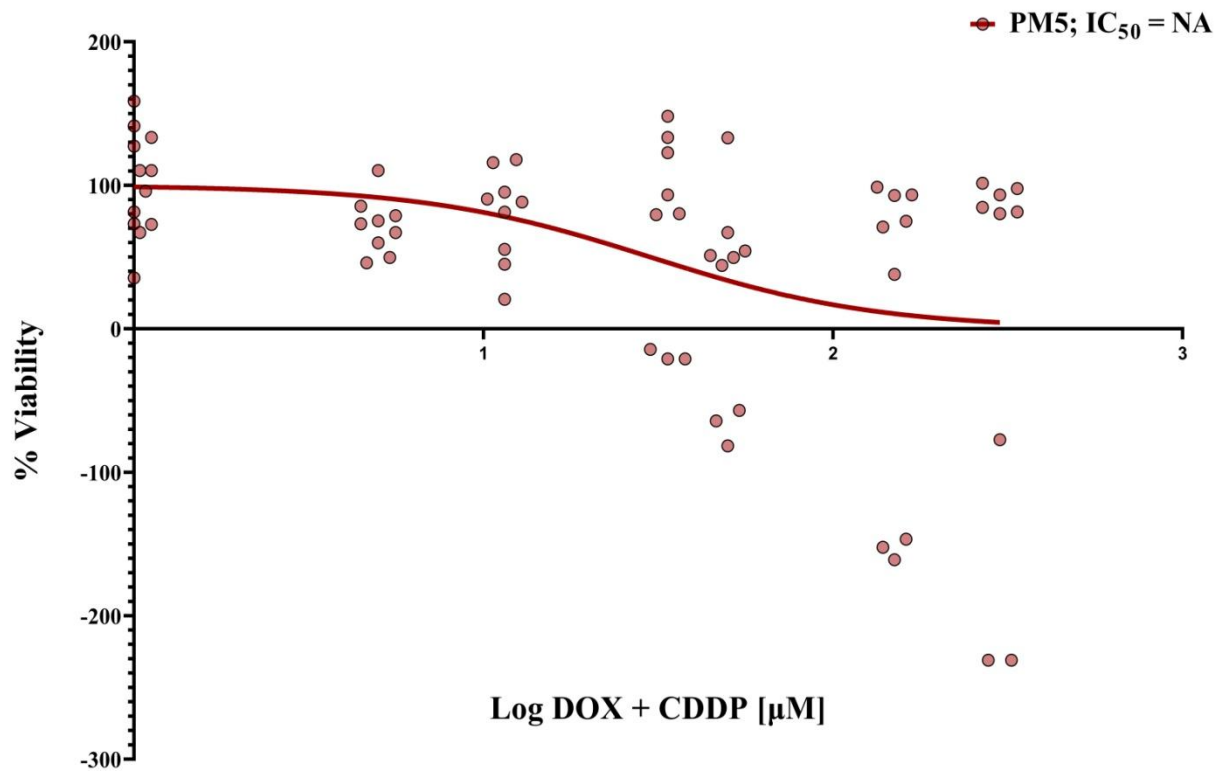


Supplementary Figure S3

HIPEC treatments - Scheme 3 - PM4 PDO line

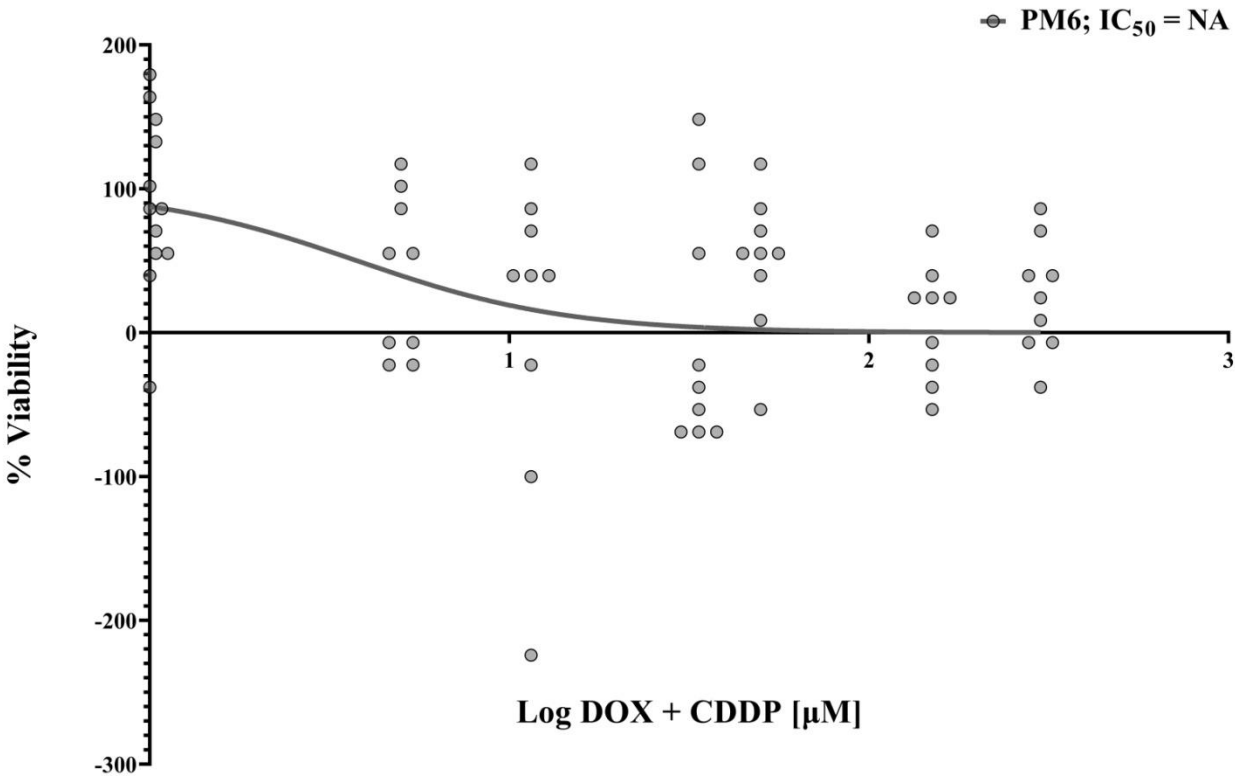


HIPEC treatments - Scheme 3 - PM5 PDO line

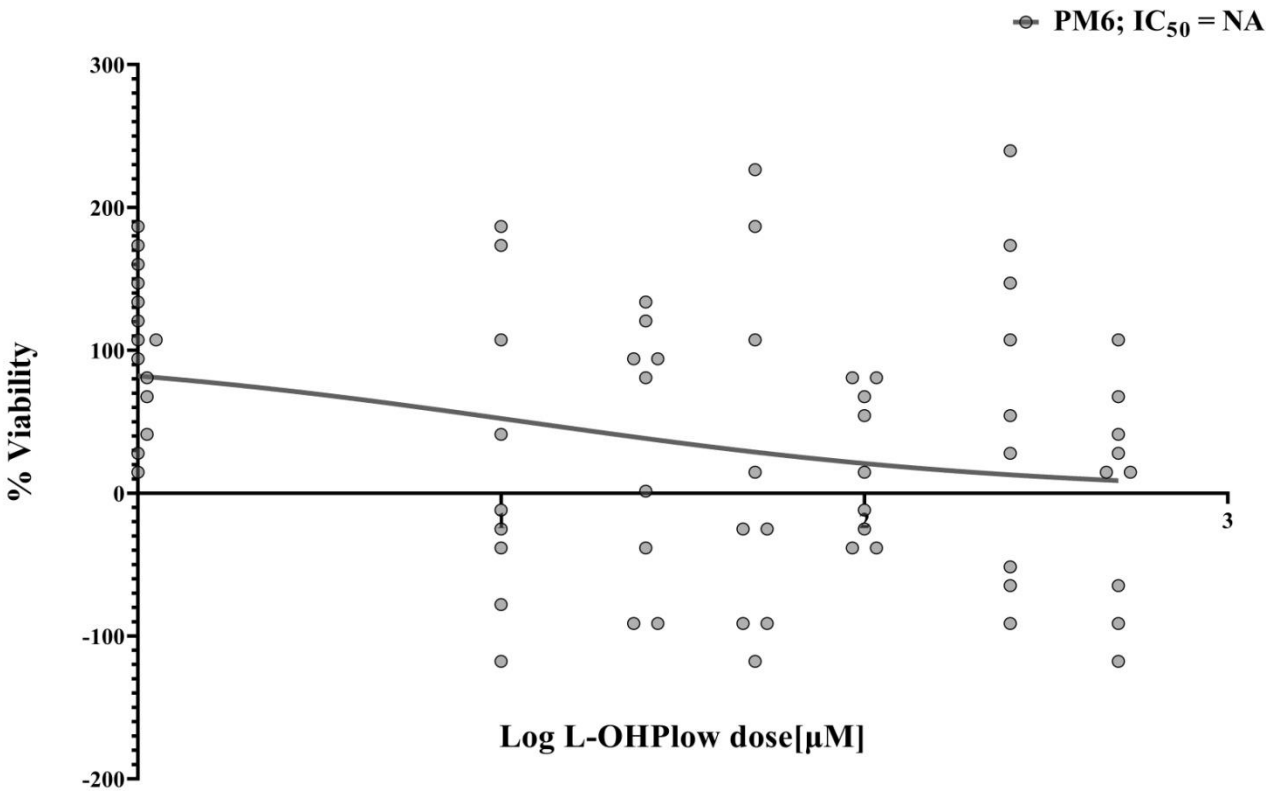


Supplementary Figure S3

HIPEC treatments - Scheme 3 - PM6 PDO line

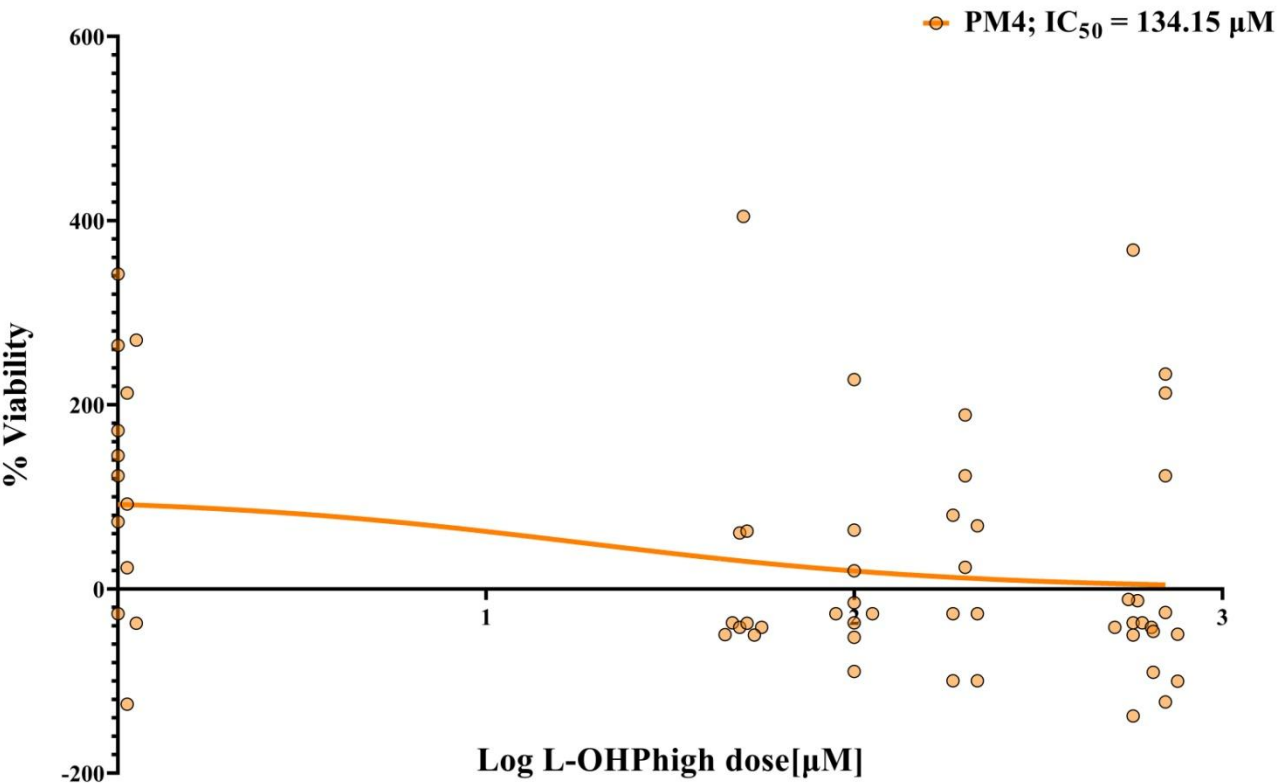


HIPEC treatments - Scheme 4 - PM6 PDO line

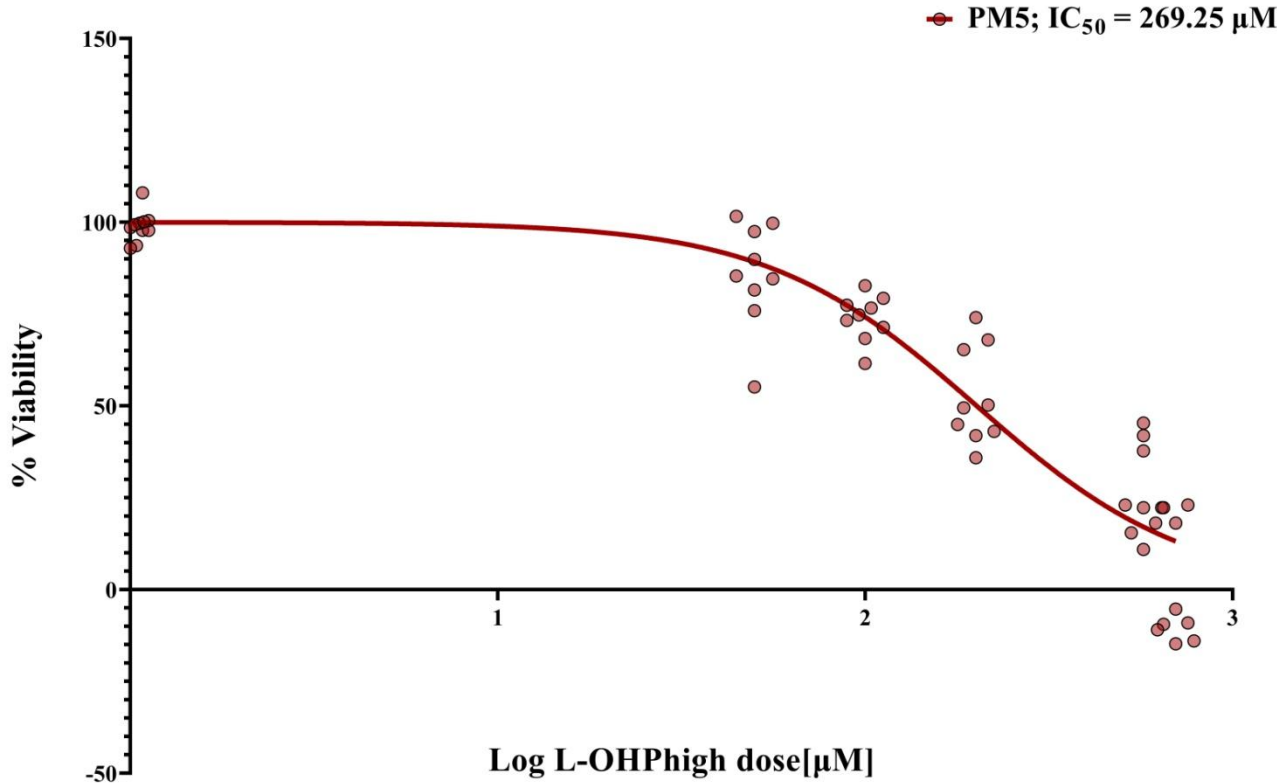


Supplementary Figure S3

HIPEC treatments - Scheme 5 - PM4 PDO line

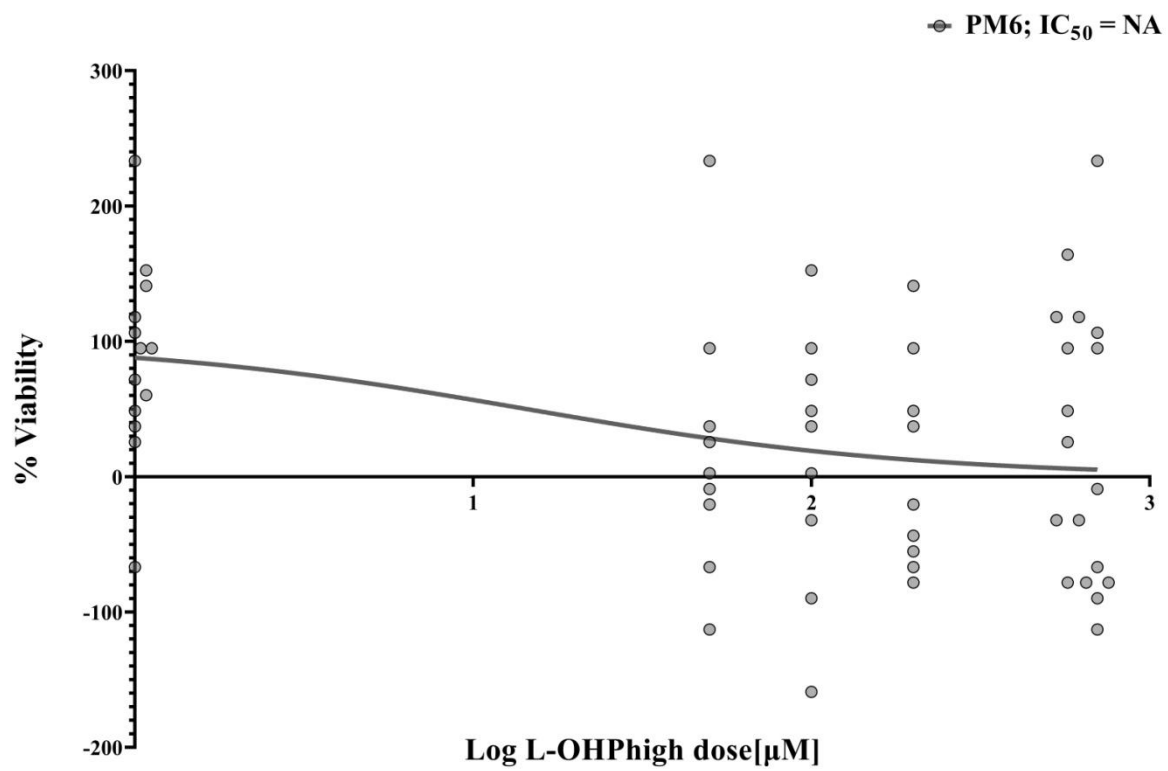


HIPEC treatments - Scheme 5 - PM5 PDO line

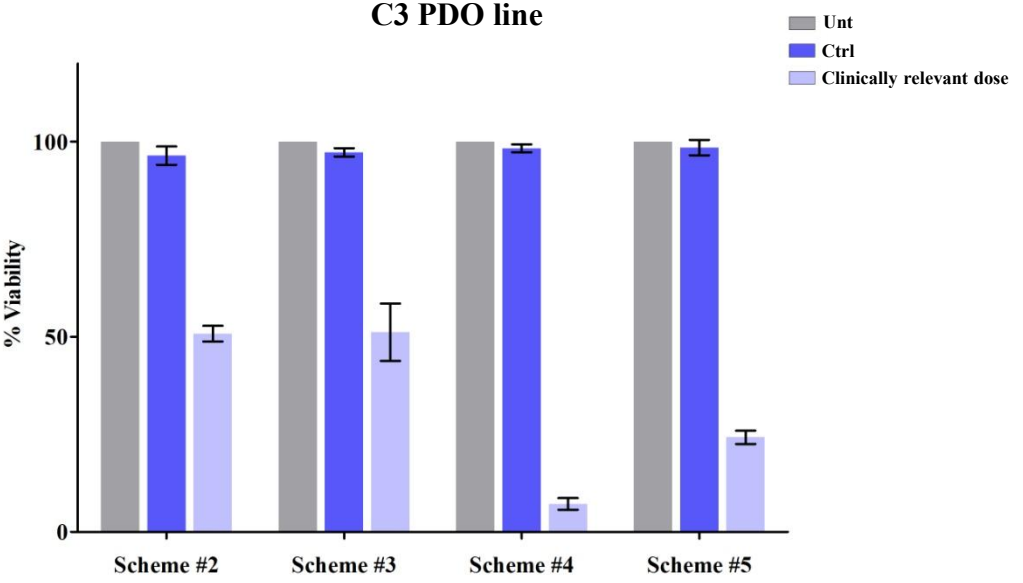
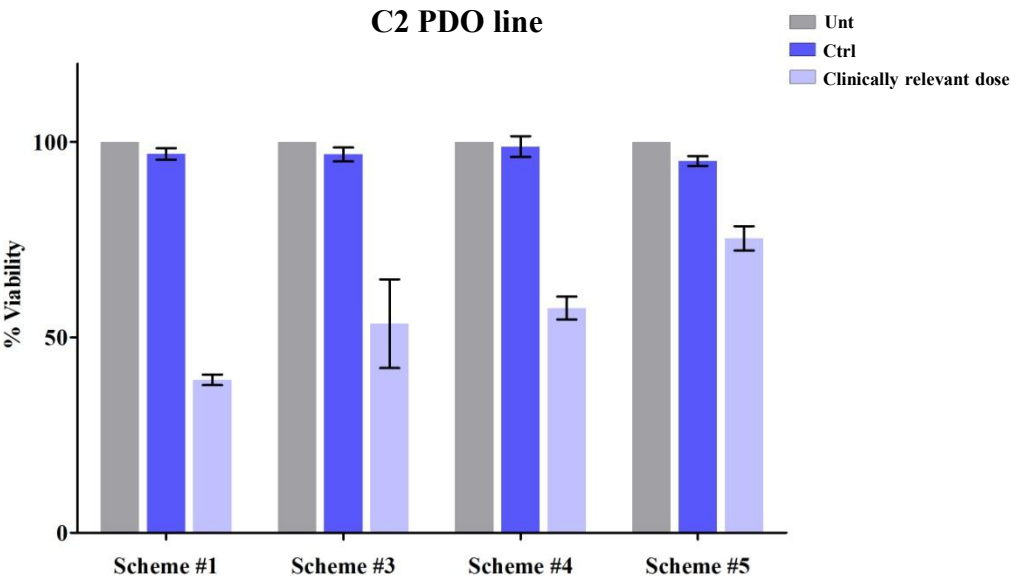
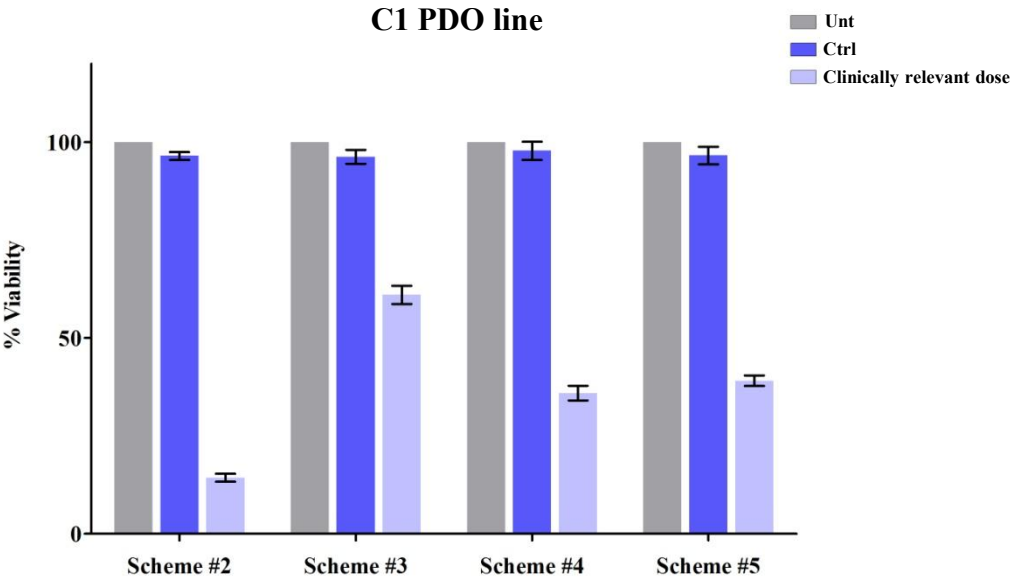


Supplementary Figure S3

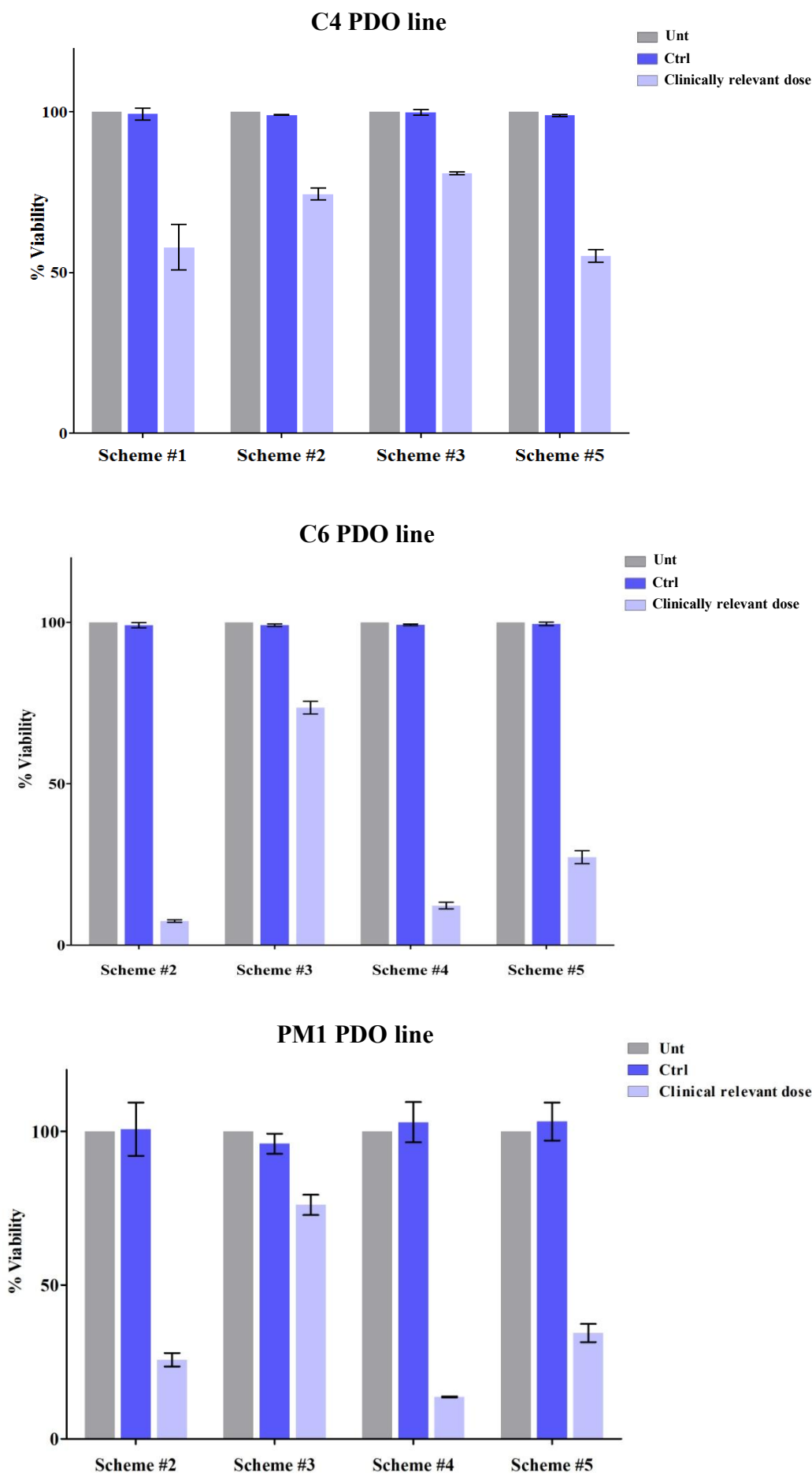
HIPEC treatments - Scheme 5 - PM6 PDO line



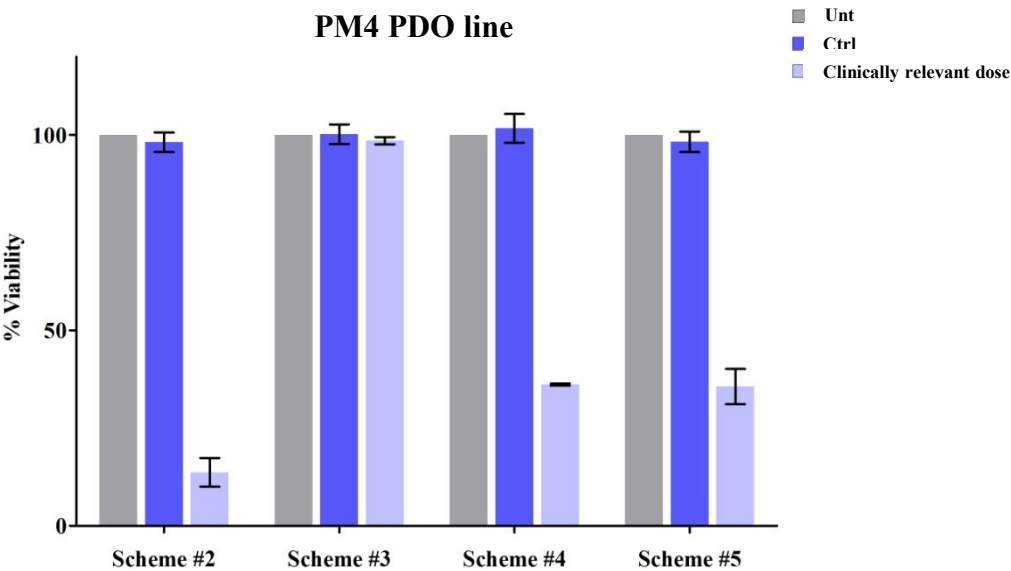
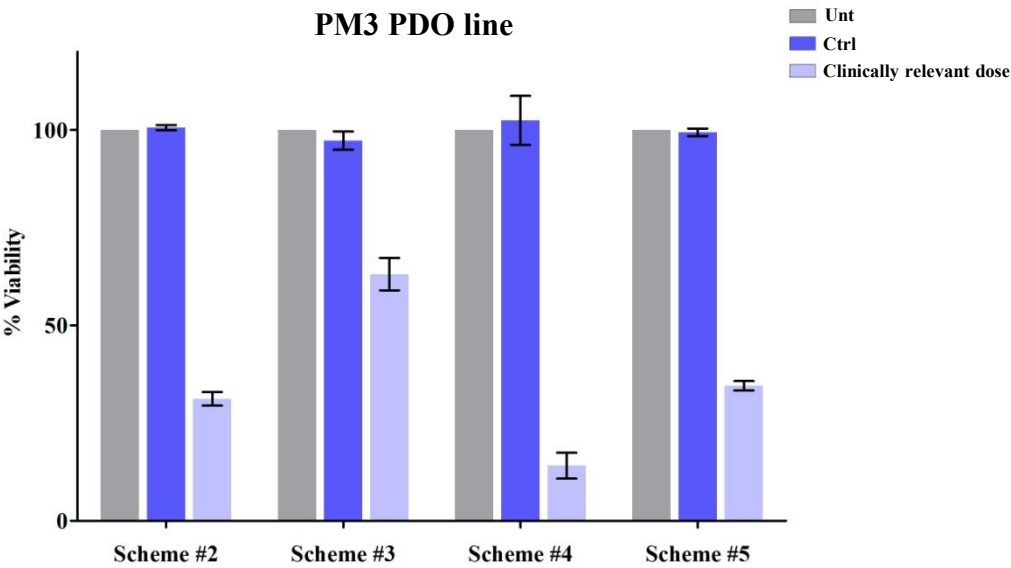
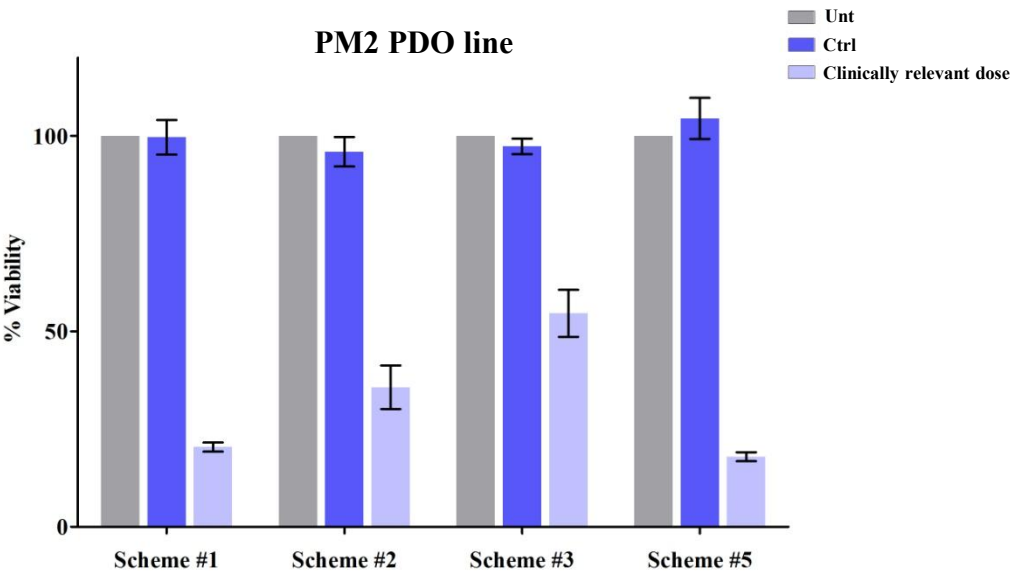
Supplementary Figure S4



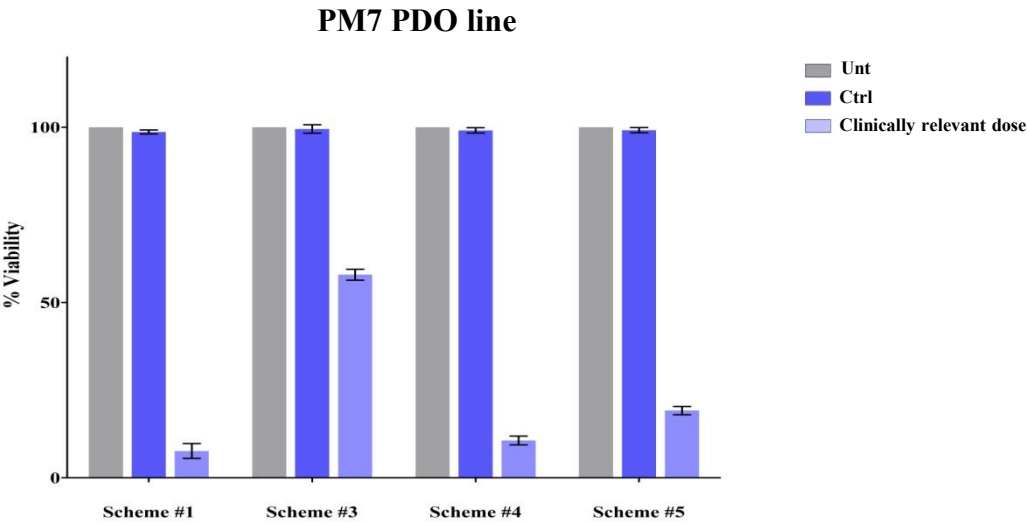
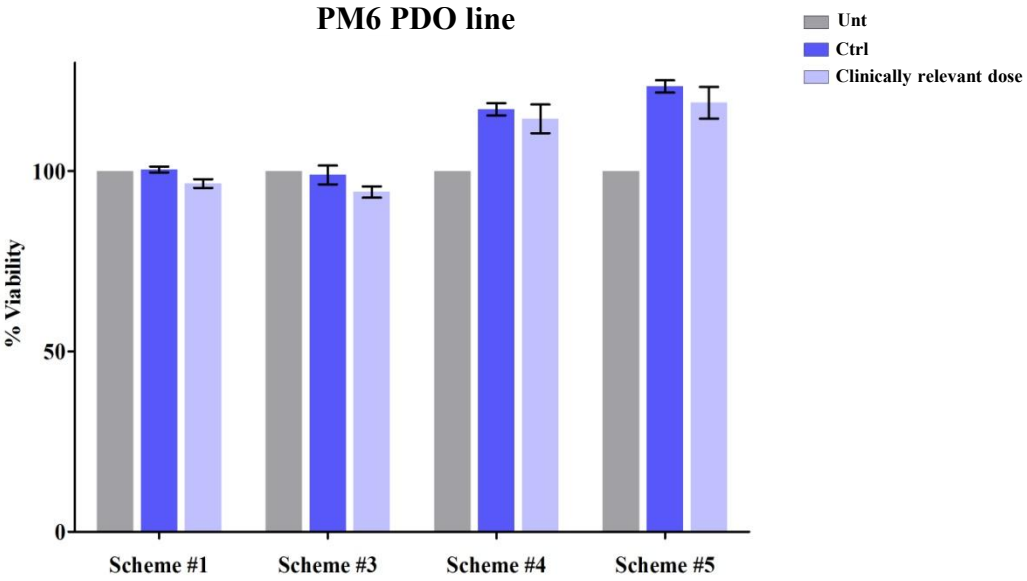
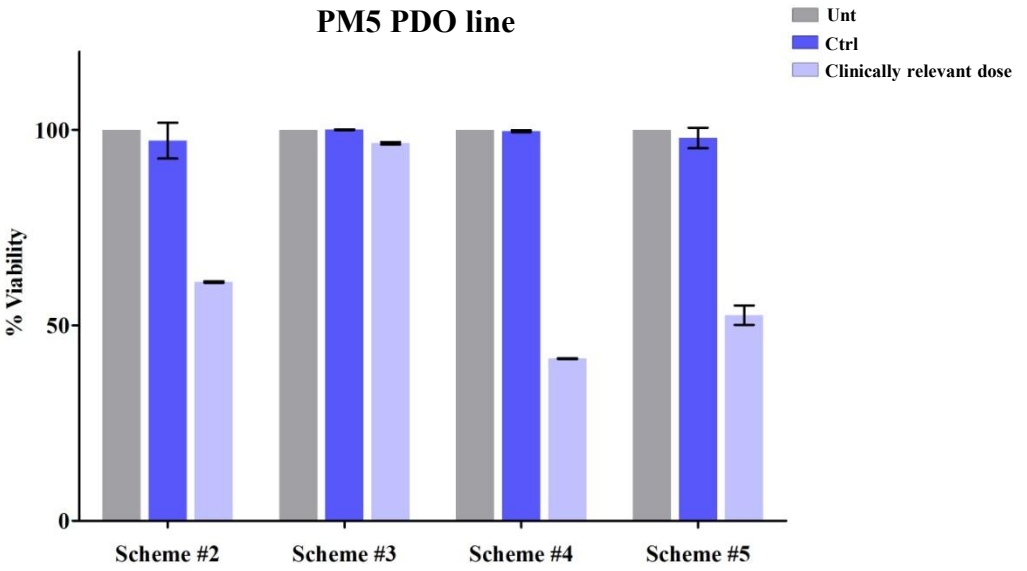
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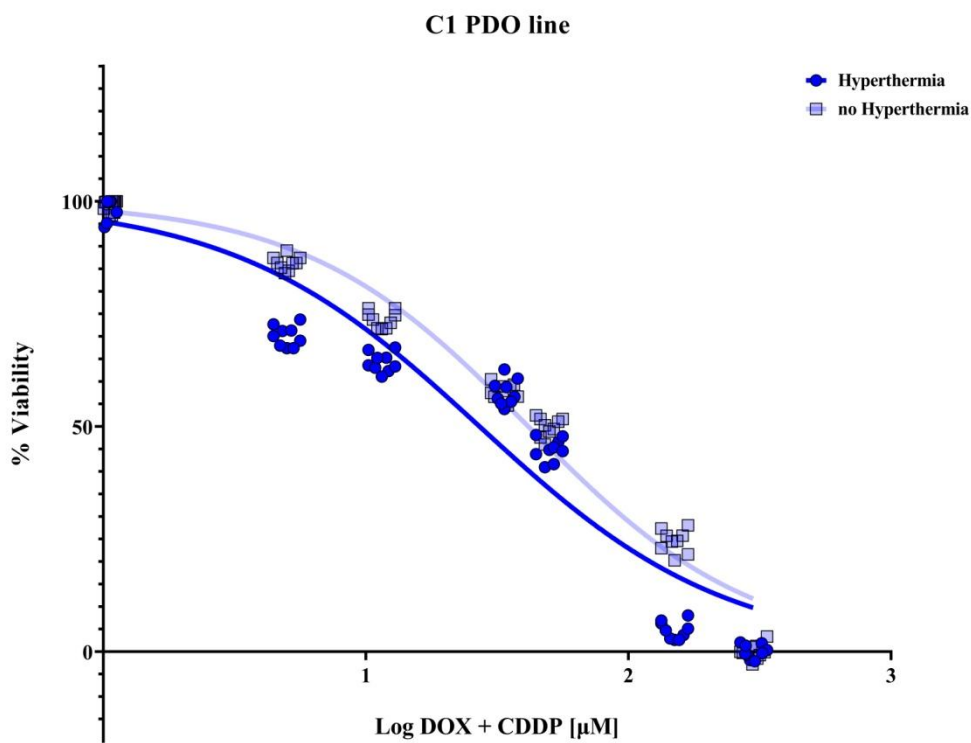
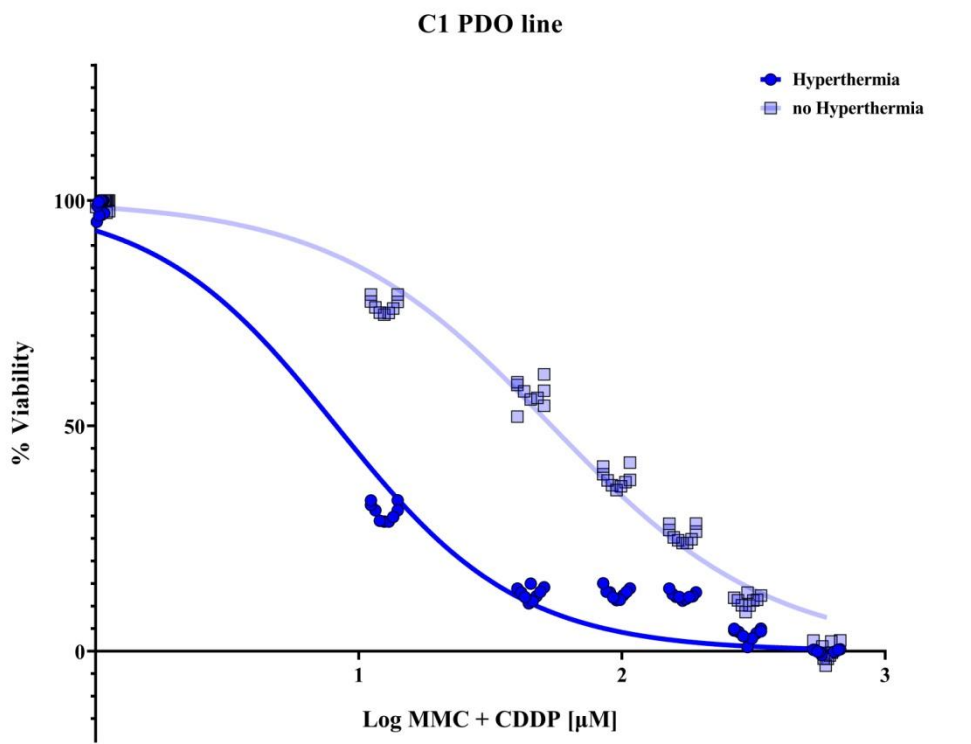
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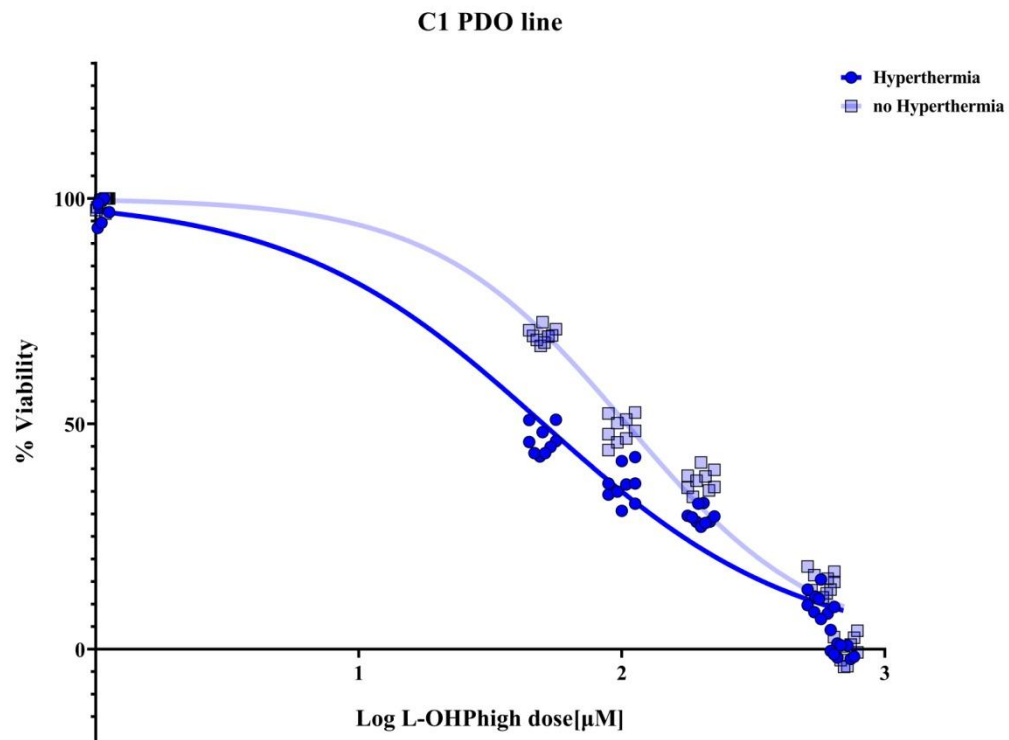
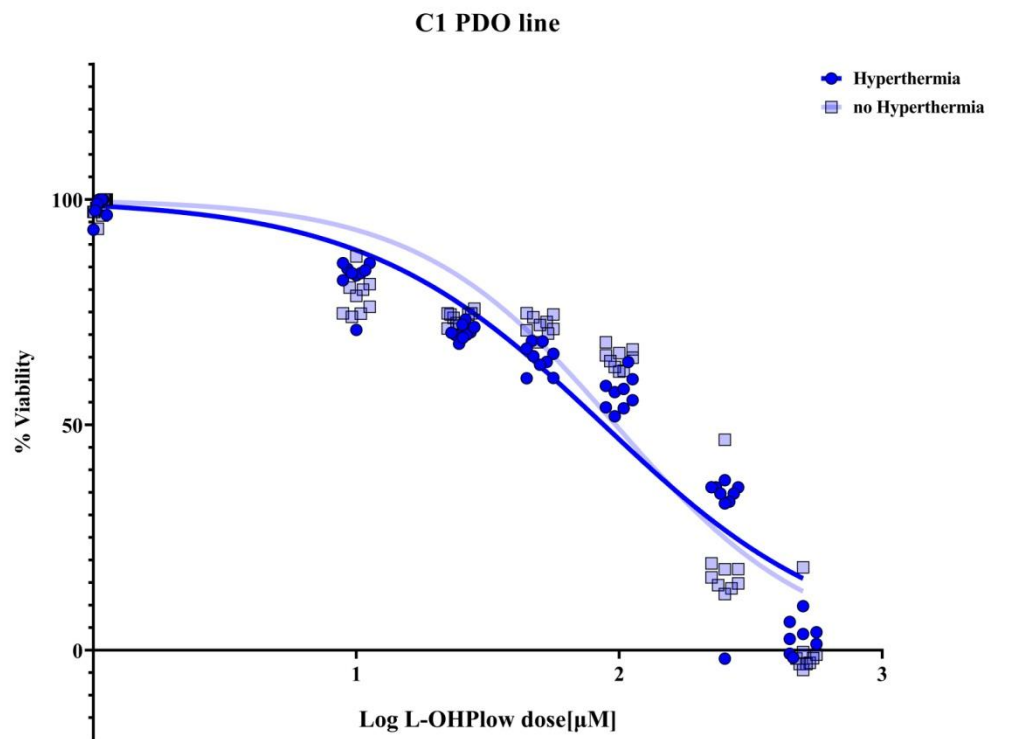
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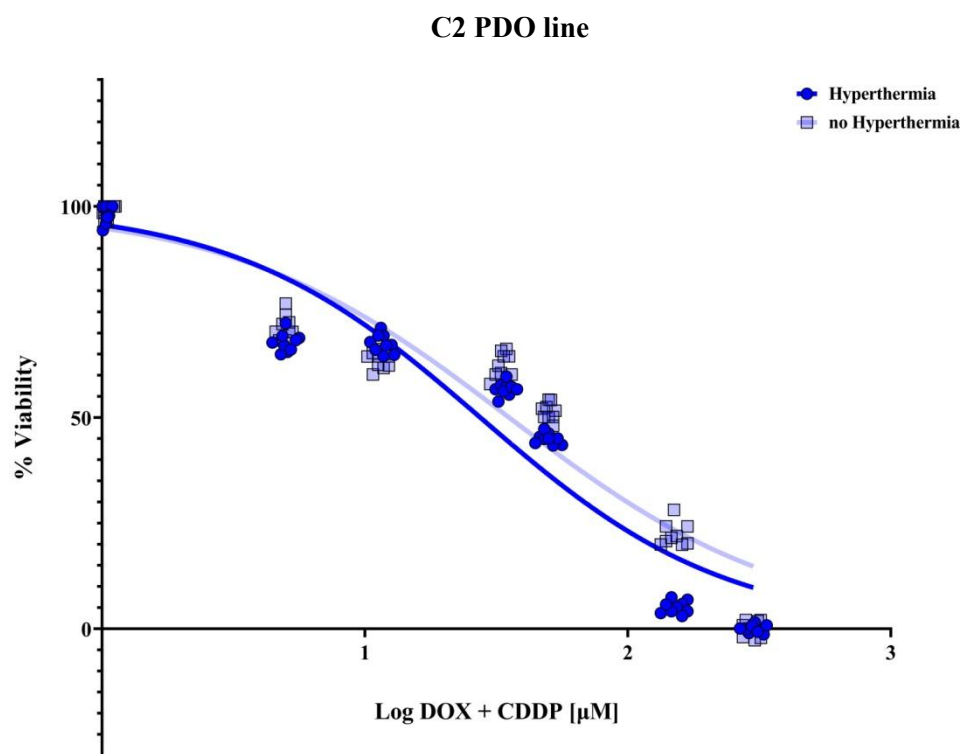
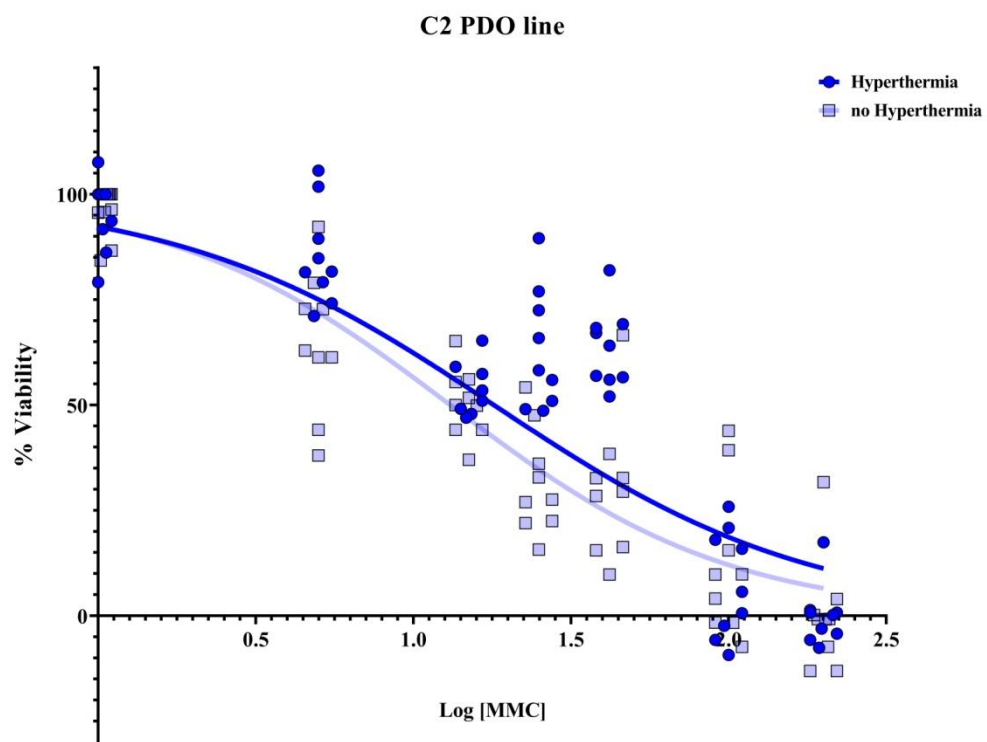
Supplementary Figure S5



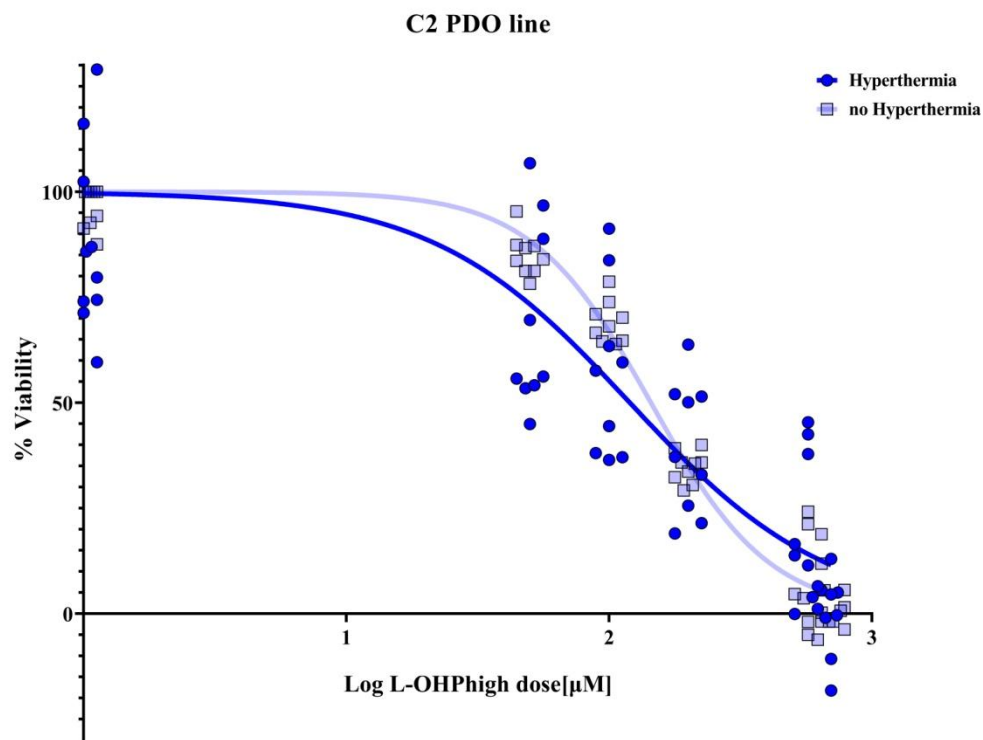
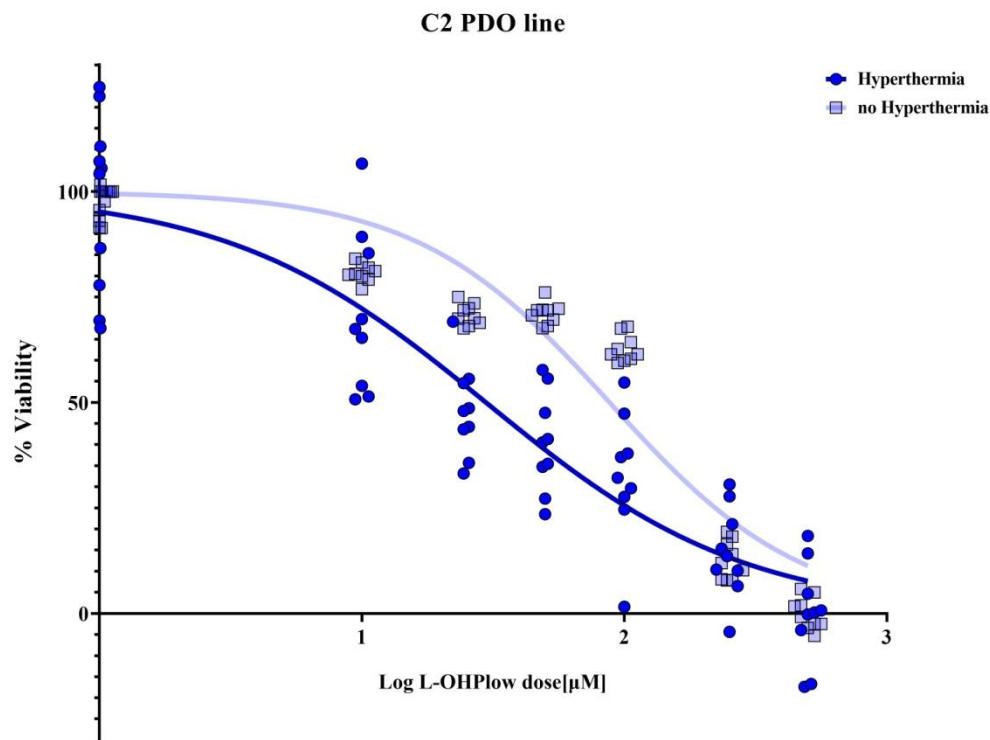
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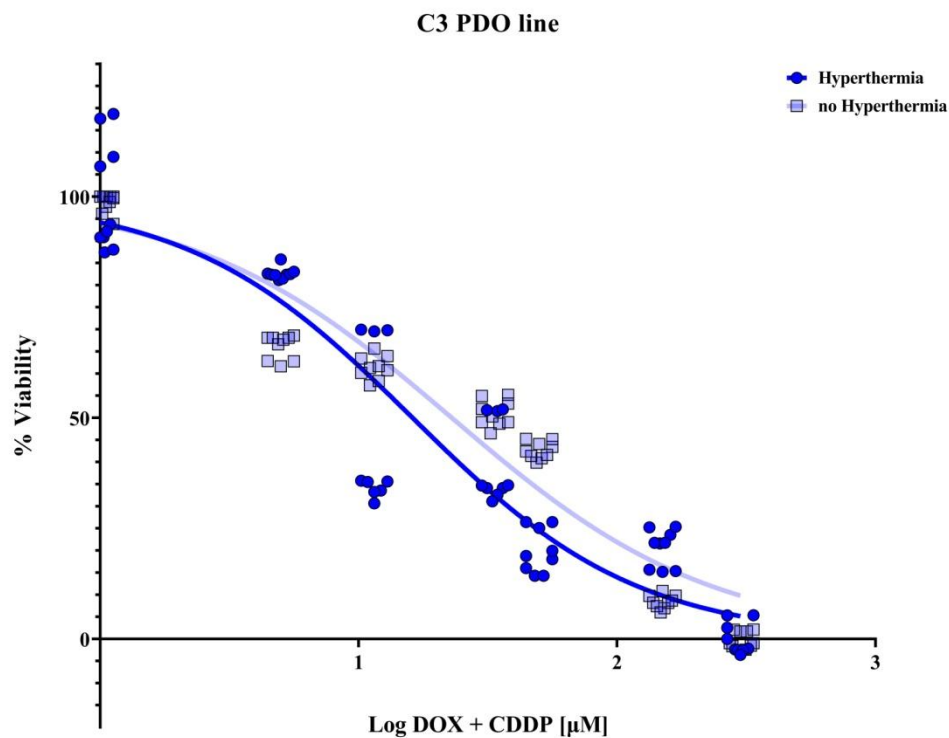
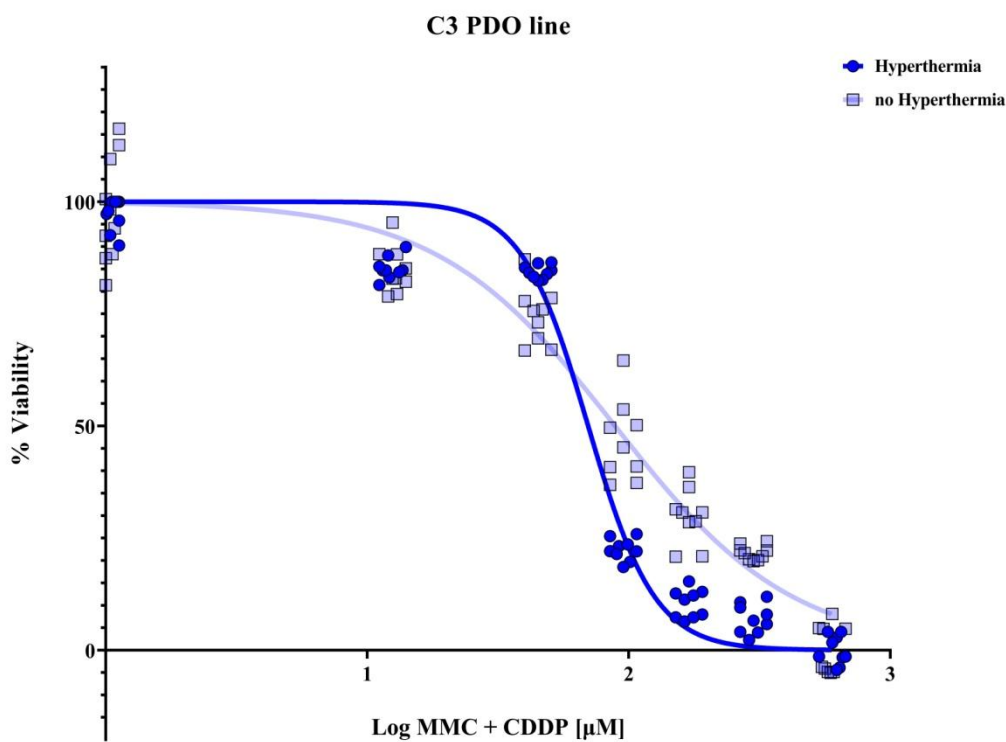
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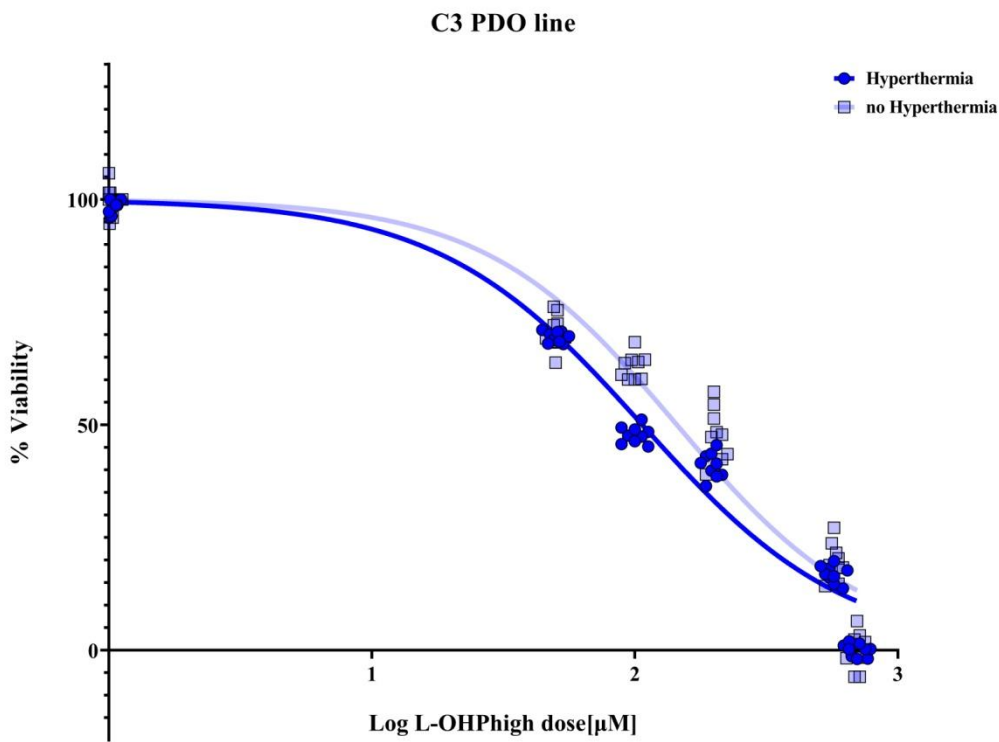
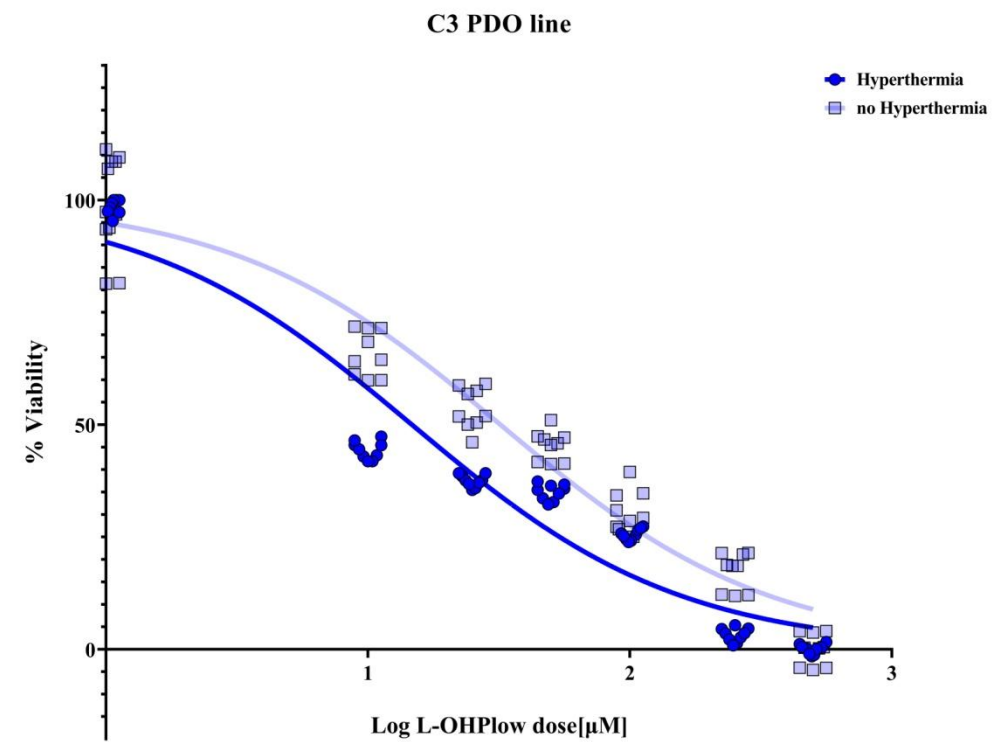
Supplementary Figure S5



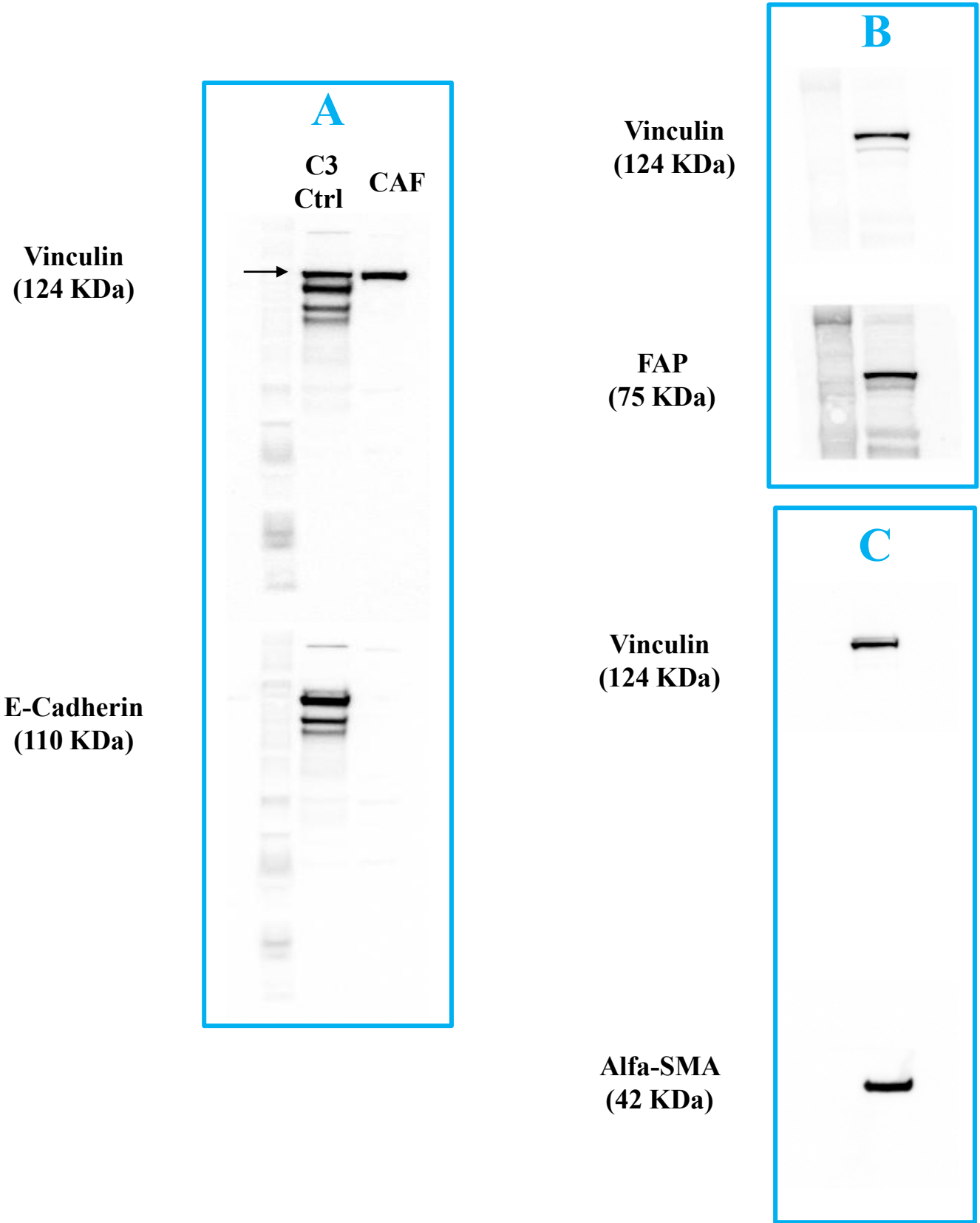
Supplementary Figure S5



Supplementary Figure S5



WB#1 CAF CRCPM



Supplementary figure legends

Supplementary Figure S1 legend: *Characterization of CRCPM-derived PDOs.* (A) Schematic representation of the protocol used to develop CRCPM-PDOs. Cells isolated from the tumor were cultured in presence of different growth factors, to mimic different niche factors conditions (WRENAS-based media). WRENAS: **W**: Wnt family member 3A; **R**: R-spondin 1 protein; **E**: Epidermal Grow Factor; **N**: Noggin protein; **A**: A83-01 – anti-p38 inhibitor; **S**: SB 202190 – anti-ROCK inhibitor

Supplementary Figure S2 legend: *PDO medium selection:* Bar plot indicating the best medium condition for each CRCPM-derived PDO cultures (C1, C2, C3, C4, C6, PM1, PM2, PM3, PM4, PM5, PM6 and PM7). The best medium formulation was determined by directly counting the number of PDO presented in each well. The experiments were performed at least in duplicate, counting three different fields per well. Differences among different media conditions were evaluated by *Student's t-test* (** $p < 0.01$; *** $p < 0.001$).

Supplementary Figure S3 legend: *PDO response to HIPEC simulation.* Dose-response curves of CRCPM-derived PDO illustrating the variation in sensitivity to MMC (scheme 1; PDO lines: C2 and PM6), MMC + CDDP (scheme 2; PDO lines: PM4 and PM6), DOX + CDDP (scheme 3; PDO lines: PM4, PM5 and PM6), L-OHP_{low-dose} (scheme 4; PDO lines: PM6) and L-OHP_{high-dose} (scheme 5; PDO lines: PM4, PM5 and PM6).

Supplementary Figure S4 legend: *PDO model systems treated with different HIPEC schemes.* Percentage of alive C1, C2, C3, C4, C6, PM1, PM2, PM3, PM4, PM5, PM6 and PM7 PDO cells after HIPEC treatments, measured as chemiluminescent signal of the concentration of intracellular ATP. PDO were treated with drug concentration corresponding to the calculated clinical relevant dose. Data are presented as median and SD and the experiments were performed in triplicate. UNT: PDO treated with media only at 42.5 °C; CTRL: PDO treated with 0.1 % of physiological solution at 42.5 °C.

Supplementary Figure S5 legend: Dose-response curves of CRCPM-derived PDO (C1, C2 and C3) illustrating the variation in sensitivity to MMC (scheme 1), MMC + CDDP (scheme 2), DOX + CDDP (scheme 3), L-OHP_{low-dose} (scheme 4) and L-OHP_{high-dose} (scheme 5) in hyperthermia and non-hyperthermia conditions.

Supplementary Figure S6 legend: *CRCPM-derived CAF characterization:* Immunoblots of (A) E-cadherin, (B) FAP, (C) and α -SMA protein expression levels in CRCPM-derived CAF lysates. Vinculin was used as loading control. Uncropped images were reported.