Supplementary Figures

Supplementary Figure S1. *In vitro* kinase assays demonstrate selectivity of ICEC0942 for CDK7. *A,* The structures of ICEC0942, BS-181 and BS-194. Mean IC_{50} values for three replicate experiments are shown (SD = standard deviation). *B,* Screening of a 117 kinase panel with 10 μM ICEC0942 was performed by the International Centre for Kinase Profiling (http://www.kinase-screen.mrc.ac.uk/). Results of duplicate assays are shown, error bars representing standard errors of the mean.

Supplementary Figure S2. Analysis of ICEC0942 treatment of MCF7 cells. *A,* ICEC0942 (1 μM) was added to MCF7 cells in culture. Cell lysates prepared after 24 hours were immunblotted. Also, immunoblotted were lysates prepared MCF7 cells treated for 24 hours with ICEC0942 at the concentrations shown. *B*, Percentage of apoptotic MCF7 cells 24 hours following addition of ICEC0942, determined by Annexin V and propidium iodide staining and flow cytometric analysis. Results of three independent experiments are shown. *C,* FACS analysis of asynchronous cultures of MCF7 cells, 24 hours after addition of ICEC0942 at the indicated concentrations. Tabulated are the percentage of cells in G1, S and G2/M phases determined from flow cytometric analysis of four independent experiments. Pair-wise comparisons of cells in each phase of the cell cycle relative to the vehicle control was performed using the unpaired t-test, *p*-values are shown.

Supplementary Figure S3. Cell Cycle analysis of MCF7 cells treated with ICEC0942. *A*, MCF7 cells were arrested in G0/G1, S-phase, or in G2/M. *B*, Cells were released from block in G1, S-phase or G2/M by washing and replenishment with fresh medium supplemented with ICEC0942. Cells were processed for flow cytometric cell cycle analysis at 0, 4, 8, 12, 24 and 48 hours following ICEC0942 addition. Bar charts show the proportion of cells in G1, S and G2/M phases of the cell cycle for each treatment. Cell cycle profiles for asynchronously growing cells are shown for comparison. The “0 hours” samples show the cell cycle distribution of MCF7 cells following treatment to enrich for cells in G1, S and G2/M phases at the point of addition of

ICEC0942 to the remaining cell cultures. *C*, MCF7 lysates in Supplementary Figure S3A were immunoblotted for CHK2, or CHK2 phosphorylation at Thr68 and Ser516. *D*, Cell lysates prepared from MCF7 cells treated for 6 hours with etoposide, which activates CHK2, in the presence or absence of ICEC0942, were immunoblotted as above.

Supplementary Figure S4. ADME analysis, oral bioavailability and distribution of ICEC0942. *A,* Results of in vitro pharmacology and physical properties. [a]24 hour turbidimetric assay. [b]$LogD_{7.4}$ was determined from addition of octanol to ICEC0942 prepared in buffer at pH 7.4. [c]Caco2 apical to basal (A-B) P_{aap} value of 0.54×10^{-6} cm/s, indicative of reasonable absorption. Atenolol, which has human absorption = 50%, used as a control in this assay, had P_{aap} = 0.35×10^{-6} cm/s. However, the B-A/A-B ratio for ICEC0942 indicates a potential efflux liability. [d]Permeation of ICEC0942 across an artificial hexadecane membrane, quantified by LC-MS/MS after a five-hour incubation at room temperature. *B, In vitro* stability assay was performed by incubation of 3 μM ICEC0942 with purified pooled human (male and female) liver microsomes, or pooled male CD1 mouse microsomes for 0, 5, 15, 30 and 45 min. followed by LC-MS/MS determination of ICEC0942 remaining. For determining hepatocyte stability, ICEC0942 (3μM) was incubated with human hepatocytes. Samples at 0, 5, 10, 20, 40 and 60 min, were used for LC-MS/MS determination of remaining ICEC0942. *C,* Shown are IC_{50} (μM) for inhibition of cytochrome P450 enzymes. *D,* Single dose intravenous (IV), subcutaneous (SC) or oral gavage (PO) administration of ICEC0942 at 10 mg/kg in CD1 male mice (n=3). ICEC0942 was prepared in 5%DMSO/PBS. *E,* ICEC0942 concentration over time. *F,* Oral bioavailability of ICEC0942 was calculated at 30%. *G,* Metabolite analysis by liquid chromatography-mass spectrometry of plasma recovered 2 and 4 hours following a single PO administration of ICEC0942 (100 mg/kg). *H-K,* ICEC0942 plasma concentration over time, following single PO administration at a concentration of 3, 10, 30 or 100 mg/kg in male CD1 mice. ICEC0942 was prepared in 5%DMSO/PBS and administered by oral gavage. Results for 3 independent animal groups is shown. *I,* Cmax vs dose administered. *J,* ICEC0942 $AUC_{t0\text{-}12h}$ vs administration dose. *K,* Mean values (n=3) for the study are shown. In

the mouse (CD1/male) following oral administration of ICEC0942 (5%DMSO in PBS) median tmax was unaffected over the range of doses and typically occurred at 2 h. *J, K,* Exposure increases in a linear manner between 10-100 mg/kg, as demonstrated by the good correlation between dose and exposure (r^2 = 0.997).

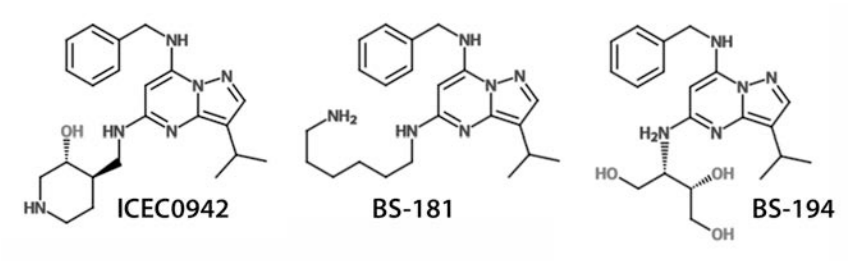
Supplementary Figure S5. Oral administration of ICEC0942 inhibits cyclin-dependent kinase substrate phosphorylation in mouse peripheral blood mononuclear cells (PBMC). Mouse PBMCs were collected 6 h following dosing of female nude mice by oral gavage at the dose levels shown. PBMCs were incubated with the appropriate primary antibodies, followed by fluorescently labelled secondary antibodies and flow cytometric analysis (n=3) to determine the status of PolII Ser2 and Ser5 phosphorylation. Error bars represent the standard errors of the mean (* = p<0.01 relative to the DMSO treated (0) controls, determined using t-test analysis).

Supplementary Figure S6. Pharmacokinetic determination of ICEC0942 plasma and tumor concentrations at different doses and over time. *A,* Plasma, tumor and liver concentrations of ICEC0942 was determined for 3 mice treated with 100 mg/kg ICEC0942 for 13 days, from the study in Fig. 4. Additionally, plasma, liver and tumor levels of mice (n=3) with established HCT116 tumors (100-200 mm^3) taken 6 hours following first administration of 50 mg/kg or 100 mg/kg ICEC0942 is shown. ICEC0942 plasma concentration is reported as μg/mL plasma. ICEC0942 concentrations in the tumor and liver are given as μg (ICEC0942)/g tissue. Standard deviations are shown in brackets. *B,* Nude mice with established HCT116 tumors (100-200 mm^3) were treated with a single PO administration of 100 mg/kg ICEC0942. Animals were sacrificed at 1, 6, 12, 18, 24, 36 and 48 h after dosing. At each time point blood and tumors were sampled for ICEC0942 remaining by LC-MS/MS. The results of three mice at each time point, are shown. A single 100 mg/kg dose of ICEC0942 was administered PO to male CD1 mice and plasma levels of ICEC0942 were determined (n=3 for each time point).

Supplementary Figure S7. Combinatorial inhibition of breast cancer cells with a combination of ICEC0942 + anti-estrogens. *A-B,* MCF7 cells were cultured in the presence of ICEC0942 (100 nM), with or without 1 nM tamoxifen or faslodex over a 9-day growth experiment. Cell number was assessed using the SRB assay and is shown relative to growth for day 0 ±SEM (n=5). ANOVA analysis for multiple comparison was performed and p-values for the key treatment pairs is shown. *C,* Representative IHC images of tumors recovered at day 15 following oral administration of 50 mg/kg/day ICEC0942 and 100 µg tamoxifen citrate daily over a 15-day period, plotted in Fig. 5C. *D,* Animal growth was measured over the course of the treatments. *E*, Biochemical markers of liver and kidney (n=4). Serum was collected 6 hours after the last dose at the end of the study (day 15). *F*, Haematological profiles in animals bearing MCF7 tumour xenografts treated with ICEC0942. Blood was collected 6h after the last dose at the end of the study.

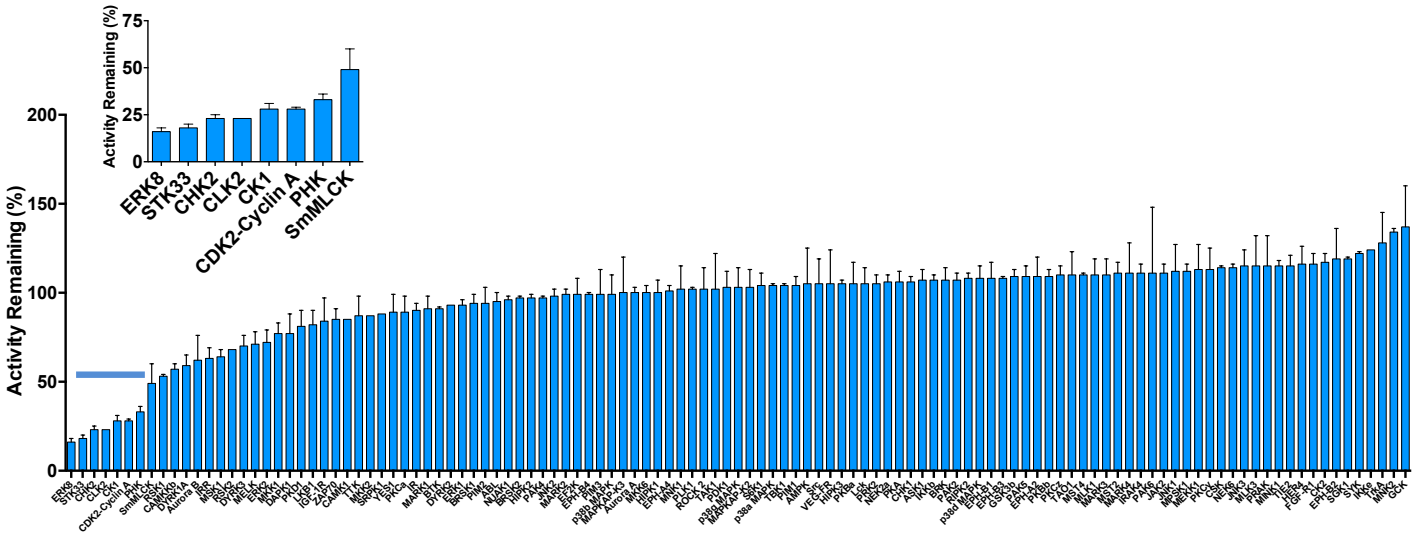
Supplementary Figure S1

A



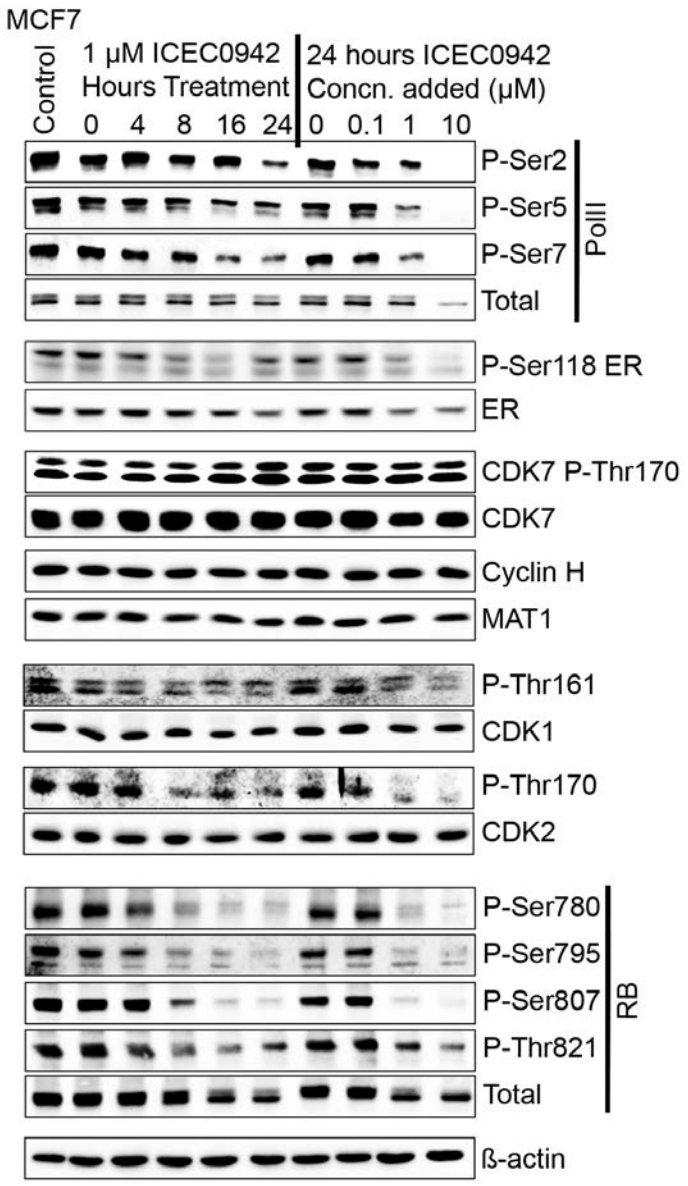
	ICEC0942 IC50 (μM) [SD]	BS-181 IC50 (μM) [SD]	BS-194 IC50 (μM) [SD]
CDK1	1.8 [0.05]	8.1 [0.6]	0.03 [0.01]
CDK2	0.62 [0.06]	0.88 [0.08]	0.003 [0.001]
CDK4	49.0 [0.3]	33.0 [1.5]	20.0 [1.3]
CDK5	9.4 [0.1]	3.0 [0.5]	0.03 [0.006]
CDK6	34.0 [0.8]	47.0 [4.0]	35.5 [1.3]
CDK7	***0.04 [0.02]***	***0.021 [0.002]***	***0.25 [0.04]***
CDK9	1.2 [0.02]	4.2 [0.5]	0.09 [0.01]

B

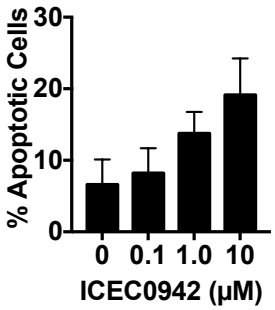


Supplementary Figure S2

A

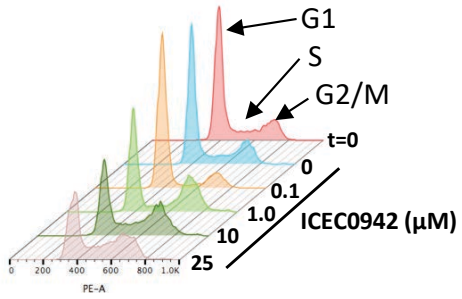


B



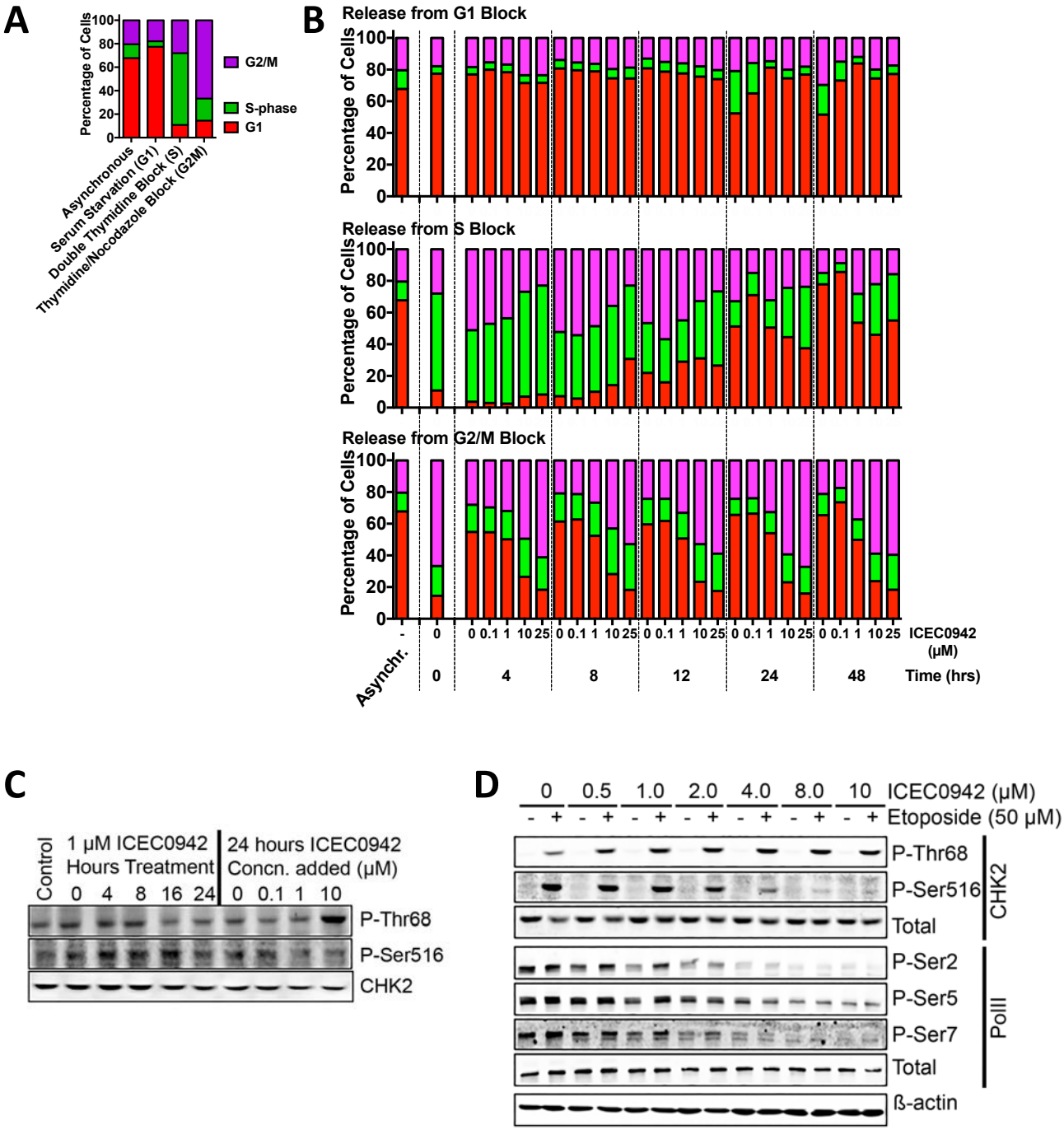
C

Asynchronous MCF7 Cells



	G1			S-phase			G2/M		
ICEC0942 (μM)	Percent of cells			Percent of cells			Percent of cells		
	Mean	STDEV	p-value	Mean	STDEV	p-value	Mean	STDEV	p-value
0	50.9	3.5		19.7	4.1		29.3	2.3	
0.1	61.9	4.6	0.009	14.1	2.4	NS	24.1	4.1	NS
1.0	48.4	3.6	NS	11.0	3.6	0.018	40.6	1.5	0.0002
10	42.8	4.6	NS	18.3	3.9	NS	38.9	2.9	0.002
25	42.5	4.6	NS	21.7	5.1	NS	35.8	2.9	0.014
n=4 independent experiments									

Supplementary Figure S3



Supplementary Figure S4

A

In vitro pharmacology/Physical properties	
Aqueous solubility (PBS pH7.4) [a]	>100 µM
LogD 7.4 (reverse phase HPLC) [b]	1.88
Plasma protein binding (%) proein bound (test conc: 5 µM)	90.8
Caco2 B-A/A-B efflux ratio (TC7, pH 6.5/7.4, test conc: 10 µM)	86.5 [c]
PAMPA (Papp 10-6 cm/s) [d]	1.18
K+ channel – hERG (automated patch clamp) test hERG expressing CHO-K1 cells (IC50 µM)	>25

B

ICEC0942 Stability (in vitro)	Human microsomes	Mouse Microsomes	Human Hepatocyte
Test Concentration (µM)	3.0	3.0	3.0
Cl_{int} (µl/min/mg protein)	13.2	9.63	5.58
$t_{1/2}$ (hours)	1.75	2.40	4.15
Parent metabolised at 45 min (%)	25.4	86.4	73.7

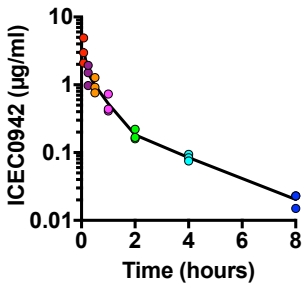
C

Cytochrome P450 inhibition	Substrate	IC_{50} (µM)
CYP2D6 Inhibition	dextromethorphan	17.7
CYP3A4 Inhibition	midazolam	5.1
CYP3A4 Inhibition	testosterone	8.8
CYP2B6 Inhibition	buproprion	21.1
CYP2C8 Inhibition	paclitaxel	>25
CYP1A Inhibition	ethoxyresorufin	>25
CYP2C9 Inhibition	mephentoin	>25
CYP2C9 Inhibition	tolbutamide	>25

D

	IV	SC	PO
Dose (mg/kg)	10	10	10
C_{max} (µg/mL)		1.7	0.2
C_0 (µg/mL)	5.4		
T_{max} (hr)		0.1	2.0
AUC to Last (µg-hr/mL)	2.3	2.7	0.6
$t_{1/2}$ (hr)	1.9		
V (mL)	365.4		
Total CL (mL/hr)	131.0		
Total CL (mL/min/kg)	77.6		
Last Time point	8.0	24.0	6.7
MRTINF (hr)	1.4	3.1	3.9
Vss (mL)	184.0		
AUC to 4 (µg-hr/mL)	2.1	2.2	0.5
% bioavailailibity		120	30

E



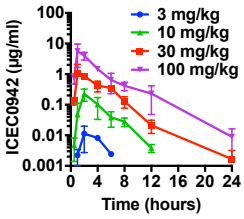
F

Blood/Plasma Ratio *	CL (blood) (ml.min/kg)	CL (plasma) (ml.min/kg)	Vd (L/kg)	t1/2 (hours)	AUCt (µg.h/ml)	C0 (µg/ml)	Clast (µg/ml)	tlast (hours)	F (%)
1.81	43	78	13	1.94	2.28	5.43	0.02	8	30
* Blood/plasma ratio determined ex vivo in male CD1 mouse blood at 1 µM ICEC0942.									
Bioavailability (F%) calculated using PO data for 10 mg/kg administration.									

G

Mass	Metabolite Name	Formula	Mass Difference from parent	Mode and m/z found	Retention time (min)	Metabolite reference	Abs area (2 hours)	Area % (2 hr)	Abs area (4 hours)	Area % (4 hr)
294	Parent	$C_{22}H_{30}N_6O$	0	395	4.93		7.15E+10	86.9	6.85E+10	86.5
293	Dehydrogenation	$C_{22}H_{28}N_6O$	-2	393	4.07	M1	8.95E+08	1.1	2.26E+08	3.0
408	Dehydrogenation + Oxidation	$C_{22}H_{28}N_6O_2$	14	409	4.75	M2	N/A	N/A	3.13E+08	0.4
410	Oxidation	$C_{22}H_{30}N_6O_2$	16	411	4.09	M3	1.12E+08	0.1	2.87E+08	0.4
410	Oxidation	$C_{22}H_{30}N_6O_2$	16	411	4.16	M4	8.91E+08	1.1	1.46E+09	1.9
410	Oxidation	$C_{22}H_{30}N_6O_2$	16	411	4.25	M5	3.04E+08	0.4	3.04E+08	0.5
410	Oxidation	$C_{22}H_{30}N_6O_2$	16	411	4.39	M6	6.60E+08	8.0	3.91E+09	5.2
424	Dehydrogenation + Di-oxidation	$C_{22}H_{28}N_6O_3$	30	425	4.13	M7	5.51E+08	0.7	4.88E+08	0.6
424	Dehydrogenation + Di-oxidation	$C_{22}H_{28}N_6O_3$	30	425	4.22	M8	4.35E+08	5.0	2.85E+08	0.4
426	Di-oxidation	$C_{22}H_{30}N_6O_3$	32	427	3.57	M9	2.39E+08	0.3	5.89E+08	0.8
426	Di-oxidation	$C_{22}H_{30}N_6O_3$	32	427	3.67	M10	7.61E+07	0.1	1.93E+08	0.3
426	Di-oxidation	$C_{22}H_{30}N_6O_3$	32	427	4.59	M11	6.06E+07	0.1	N/A	N/A
396	Gain of 2 amu	$C_{22}H_{32}N_6O$	2	397	4.32	M12	N/A	N/A	1.17E+08	0.2
398	Gain of 4 amu	$C_{22}H_{34}N_6O$	4	399	5.31	M13	2.89E+08	0.4	N/A	N/A
398	Gain of 4 amu	$C_{22}H_{34}N_6O$	4	399	5.80	M14	3.15E+08	0.4	NA	N/A
Oxidation describes a potential oxidation, epoxidation or oxide formation										

H



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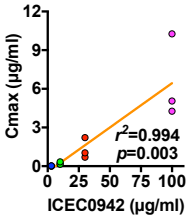

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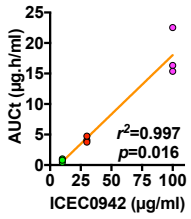
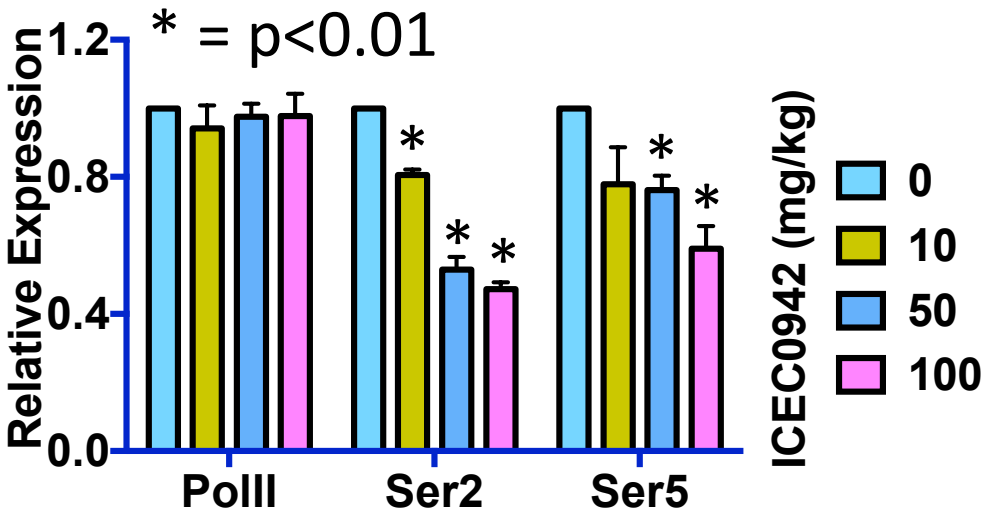


K

Dose (mg/kg)	Cmax (µg/ml)	Tmax (hours)	tlast (hours)	AUC_{t0-24h} (µg.hour/ml)	AUC_{t0-12h} (µg.hour/ml)
3	0.01	2	6	0.04	-
10	0.23	2	12	0.75	0.75
30	1.31	2	24	4.19	4.11
100	6.53	1	24	18.1	16.6

Supplementary Figure S5

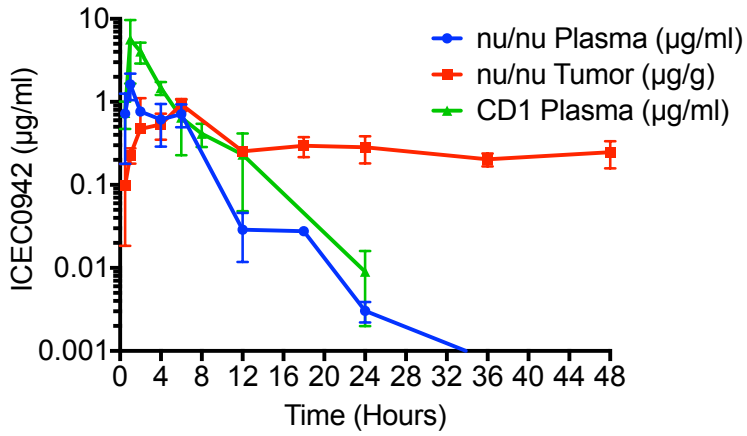


Supplementary Figure S6

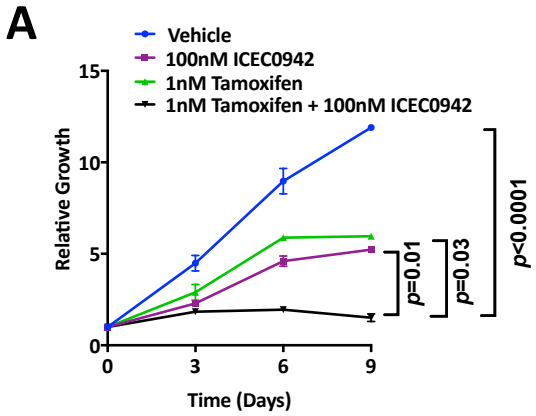
A

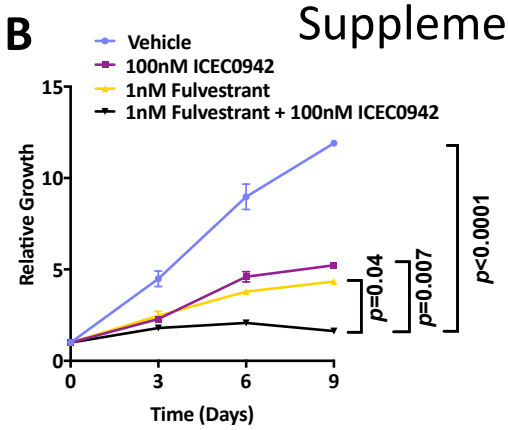
	ICEC0942 50 mg/kg		ICEC0942 (100 mg/kg/day)		
	Day 1		Day 1		
		Mean Ratio		Mean Ratio	Mean Ratio
	Concentration (µg/mL or g)	Tissue:Plasma	Concentration (µg/mL or g)	Tissue:Plasma	100: 50 mg/kg
Plasma	0.43 (0.25)	-	0.82 (0.26)	-	1.91
Liver	8.85 (3.18)	20.7	47.7 (29.6)	58.3	5.39
Tumor	3.04 (1.18)	7.1	4.13 (0.88)	5.04	1.36
			Day 13		
				Mean Ratio	Ratio
			Concentration (µg/mL or g)	Tissue:Plasma	Day 13:1
Plasma			1.64 (1.05)	-	2.01
Liver			49.3 (3.24)	30.0	1.03
Tumor			25.4 (3.24)	15.5	6.16

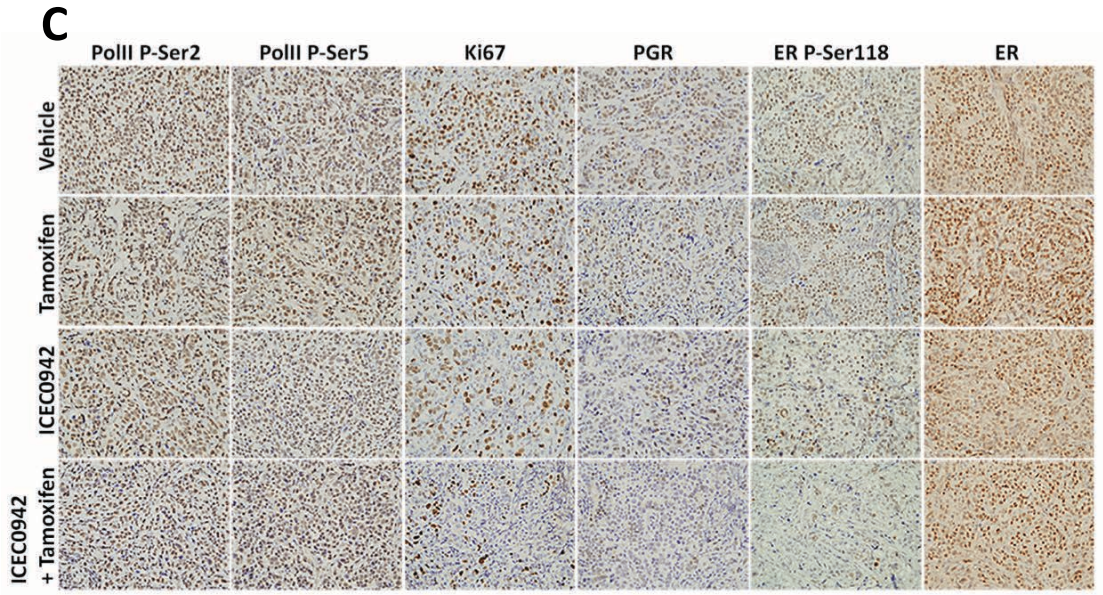
B

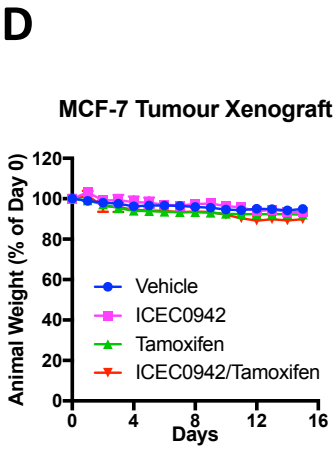


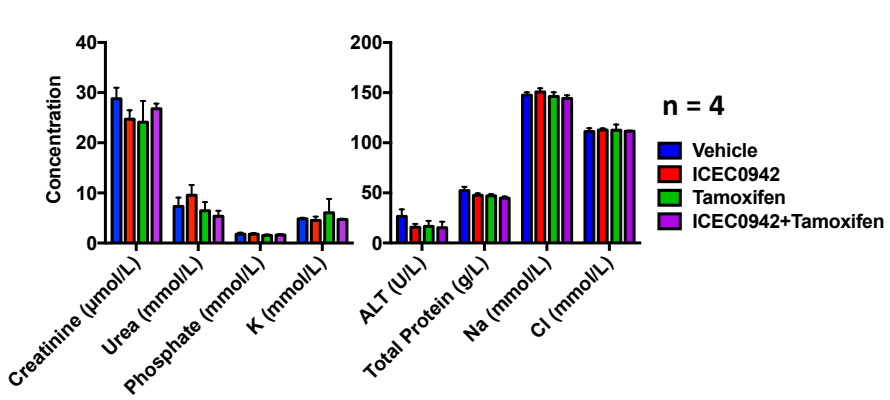
Supplementary Figure S7











F

	units	Vehicle (n=3)		ICEC0942 (n=4)		Tamoxifen (n=4)		Tamoxifen+ICEC0942 (n=4)	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Red blood cell (RBC)	10*12/L	**9.3**	0.3	**9.1**	0.7	**9.5**	0.1	**9.7**	0.4
Hemoglobin (HB)	g/dl	**15.4**	0.2	**15.5**	0.6	**15.7**	0.2	**16.2**	0.9
Hematocrit (HCT)	L/L	**0.5**	0.0	**0.5**	0.0	**0.5**	0.0	**0.5**	0.0
Mean corpuscular volume (MCV)	fL	**50.0**	0.9	**50.5**	1.5	**49.8**	0.9	**49.7**	0.7
Mean corpuscular hemoglobin (MCH)	pg	**16.7**	0.4	**17.3**	0.9	**16.6**	0.3	**16.6**	0.3
Mean corpuscular hemoglobin concentration (MCHC)	g/dL	**33.3**	0.5	**34.1**	0.7	**33.2**	0.2	**33.4**	0.1
Red cell distribution width (RDW)		**18.4**	0.2	**19.5**	1.6	**18.2**	0.2	**18.0**	0.4
Platelet (PLT)	10*9/L	**117.7**	10.3	**274.8**	48.0	**364.8**	26.3	**301.3**	46.0
White blood cells (WBC)	10*9/L	**2.5**	0.3	**3.3**	0.7	**3.5**	0.5	**4.3**	0.9
Neutrophils (NE)	10*9/L	**0.5**	0.0	**0.2**	0.1	**0.5**	0.1	**0.4**	0.2
Lymphocytes (LY)	10*9/L	**1.6**	0.3	**2.4**	0.5	**2.4**	0.3	**3.5**	0.9
Monocytes (MO)	10*9/L	**0.4**	0.2	**0.8**	0.2	**0.5**	0.2	**0.3**	0.1
Eosinophils (EO)	10*9/L	**0.0**	0.0	**0.0**	0.0	**0.0**	0.0	**0.0**	0.0
Basophils (BA)	10*9/L	**0.0**	0.0	**0.0**	0.0	**0.0**	0.0	**0.0**	0.0