Differential targets engaged by narazaciclib in comparison to the approved CDK4/6 inhibitors contribute to enhanced inhibition of tumor cell growth

Petros Kechagioglou[1], Camille Dupont[1], Hajime Yurugi[1], Ute Distler[2], Stefan Tenzer[2], Alexey Chernobrovkin[3], Kristina Riegel[1], Julianne Mooz[1], Mahil Lamber[4], Volker Dötsch[4], Stephen Cosenza[5], Steven M Fruchtman[5] and Krishnaraj Rajalingam[1]

[1]Cell Biology Unit, University Medical Center Mainz, Germany, [2]Institute of Immunology, University Medical Center Mainz, Germany, [3]Pelagobio Bioscience, Sweden, [4]Goethe University, Frankfurt, Germany, [5]Onconova Therapeutics Inc, USA

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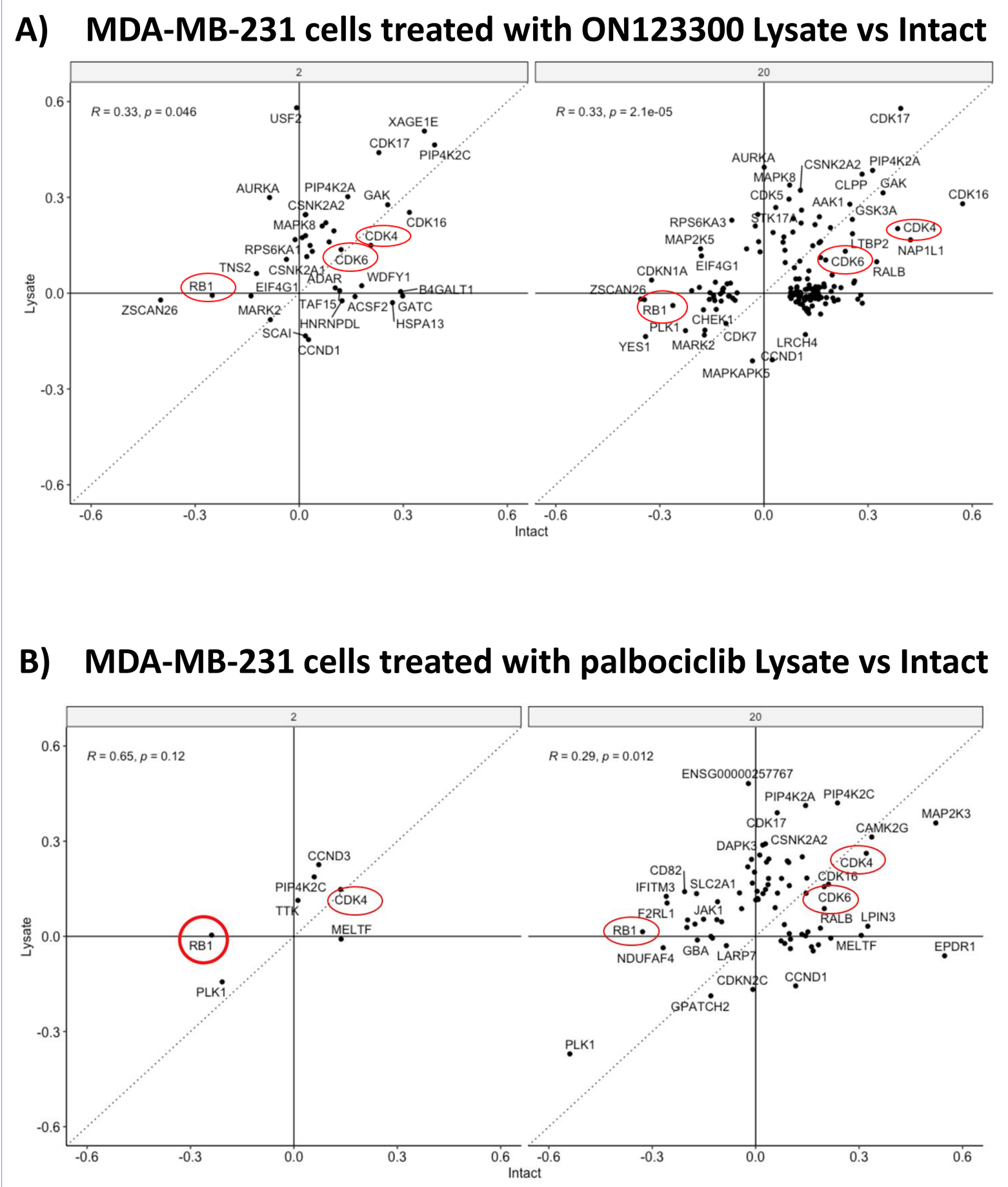
Abstract

- Narazaciclib (ON123300), a novel CDK4/6 inhibitor, designed to enhance efficacy and safety by its multi-targeted kinase inhibitor activity at low nM concentrations against CDK4/6, ARK5, CSF1R, and c-Kit.
- Narazaciclib is in Ph I trials; NCT04739293 and CXHL1900340; studying different administration regimens.
- Challenge: Despite clinical benefit, safety concerns such as neutropenia and diarrhea, and disease progression, raises a critical need to identify novel therapeutic strategies.
- Aim: Examine the efficacy of ON123300 in various breast cancer cell lines, and consolidate its mechanism of action by identifying the other targets engaged by ON123300.

Cellular Thermal Shift Assay (CETSA) profiling of MDA-MB-231 cells treated with ON123300 compared to palbociclib – Identification of cellular targets



C)

29 specific to ON 123300 in Lysates

Gene	Group
CDC42BPB	Destabilization
CHEK1	Destabilization
DGCR6	Destabilization
MAPKAPK5	Destabilization
TBK1	Destabilization
UVSSA	Destabilization
ZNF260	Destabilization
AAK1	Stabilization
ATAT1	Stabilization
AURKA	Stabilization
BMP2K	Stabilization
BUB1	Stabilization
CDK2	Stabilization
CDK5	Stabilization
CPQ	Stabilization
EPHA2	Stabilization
FKBP8	Stabilization
GSK3A	Stabilization
GSK3B	Stabilization
LIMK1	Stabilization
MAP11	Stabilization
PER3	Stabilization
SLK	Stabilization
STK17A	Stabilization
STK17B	Stabilization
TFEB	Stabilization
TGFBR2	Stabilization
USF2	Stabilization
ZCRB1	Stabilization

148 specific to ON 123300 in Intact cells

Gene	Group	Gene	Group	Gene	Group	Gene	Group
ACAA1	Destabilization	AAK1	Stabilization	GALNT7	Stabilization	MLEC	Stabilization
AIFM2	Destabilization	ADAR	Stabilization	GCAT	Stabilization	MPHOSPH8	Stabilization
ANP32B	Destabilization	AGRN	Stabilization	GSK3A	Stabilization	MRC2	Stabilization
AP2A1	Destabilization	AHCTF1	Stabilization	GSK3B	Stabilization	MRPL55	Stabilization
AP2M1	Destabilization	APLP2	Stabilization	GSKIP	Stabilization	MTG1	Stabilization
AP2S1	Destabilization	APP	Stabilization	HNRNPA0	Stabilization	NAP1L1	Stabilization
AVEN	Destabilization	ARRB2	Stabilization	HNRNPA1	Stabilization	NAPG	Stabilization
BOD1L1	Destabilization	AXL	Stabilization	HNRNPA2B1	Stabilization	NRP1	Stabilization
CDK12	Destabilization	BMP2K	Stabilization	HNRNPA3	Stabilization	PHF8	Stabilization
CDK7	Destabilization	CAVIN1	Stabilization	HNRNPC	Stabilization	PIK3C3	Stabilization
CDKN1A	Destabilization	CAVIN3	Stabilization	HNRNPL	Stabilization	PIK3R4	Stabilization
CHEK1	Destabilization	CBX5	Stabilization	HNRNPR	Stabilization	PIP4K2A	Stabilization
COG8	Destabilization	CCN1	Stabilization	HNRNPU	Stabilization	PIP4K2B	Stabilization
DECR1	Destabilization	CDC5L	Stabilization	HNRNPU	Stabilization	PIP4P2	Stabilization
EIF4G1	Destabilization	CDK17	Stabilization	HNRNPUL2	Stabilization	PRPF4B	Stabilization
FOS	Destabilization	CELF1	Stabilization	HSPA13	Stabilization	PTX3	Stabilization
FOSB	Destabilization	CERS2	Stabilization	HSPG2	Stabilization	RAE1	Stabilization
FOXK1	Destabilization	CLPP	Stabilization	IFT52	Stabilization	RBM34	Stabilization
LYN	Destabilization	CLUH	Stabilization	IGFBP4	Stabilization	RIPK1	Stabilization
LYPLA2	Destabilization	COL2A1	Stabilization	IGFBP7	Stabilization	RNF5	Stabilization
MAP2K5	Destabilization	CPD	Stabilization	ILF3	Stabilization	RRAS2	Stabilization
MAP3K20	Destabilization	CRAT	Stabilization	INCENP	Stabilization	RSF1	Stabilization
MARK2	Destabilization	CSNK2A2	Stabilization	JMY	Stabilization	SCAF1	Stabilization
MICALL2	Destabilization	CST3	Stabilization	LAMA5	Stabilization	SGPP1	Stabilization
NPM1	Destabilization	DBNDD2	Stabilization	LAMC1	Stabilization	SIK2	Stabilization
PUS7L	Destabilization	DDX21	Stabilization	LAMTOR2	Stabilization	SMU1	Stabilization
RPS6KA3	Destabilization	DHX9	Stabilization	LEO1	Stabilization	SND1	Stabilization
SART3	Destabilization	DNAJC13	Stabilization	LMAN2	Stabilization	STK10	Stabilization
SCP2	Destabilization	ECE1	Stabilization	LOXL2	Stabilization	STX5	Stabilization
SHCBP1	Destabilization	ELAVL1	Stabilization	LRCH4	Stabilization	SYNCRIP	Stabilization
SMAD1	Destabilization	ELL	Stabilization	LRPPRC	Stabilization	SYNE2	Stabilization
SNX30	Destabilization	ESYT1	Stabilization	LRRC59	Stabilization	TAF15	Stabilization
SNX6	Destabilization	FASTKD5	Stabilization	LTBP2	Stabilization	TIMP2	Stabilization
STK3	Destabilization	FBRS	Stabilization	MAMDC2	Stabilization	TTK	Stabilization
TLE5	Destabilization	FGFRL1	Stabilization	MAN2A1	Stabilization	UFL1	Stabilization
YES1	Destabilization	FUBP3	Stabilization	MATR3	Stabilization	WTAP	Stabilization
ZNF608	Destabilization	GAK	Stabilization	METTL18	Stabilization	ZFR	Stabilization

5 Common to ON 123300 in both Lysates and Intact cells

Gene	Group
CHEK1	Destabilization
AAK1	Stabilization
BMP2K	Stabilization
GSK3A	Stabilization
GSK3B	Stabilization

Figure 1. A mass spectrometry-based cellular Thermal Shift Assay (CETSA-MS) profiling of MDA-MB-231.
Both lysates and intact cells were treated with **(A)** ON123300 (2 µM and 20 µM) or **(B)** palbociclib (2 µM and 20 µM). **(C)** Table with list of the potential unique ON123300 targets compared to palbociclib ($p<0,01$).

Patients with high tumor expression of BUB1 have low survival probabilities in Breast Cancer and Uterine Corpus Endometrial Carcinomas

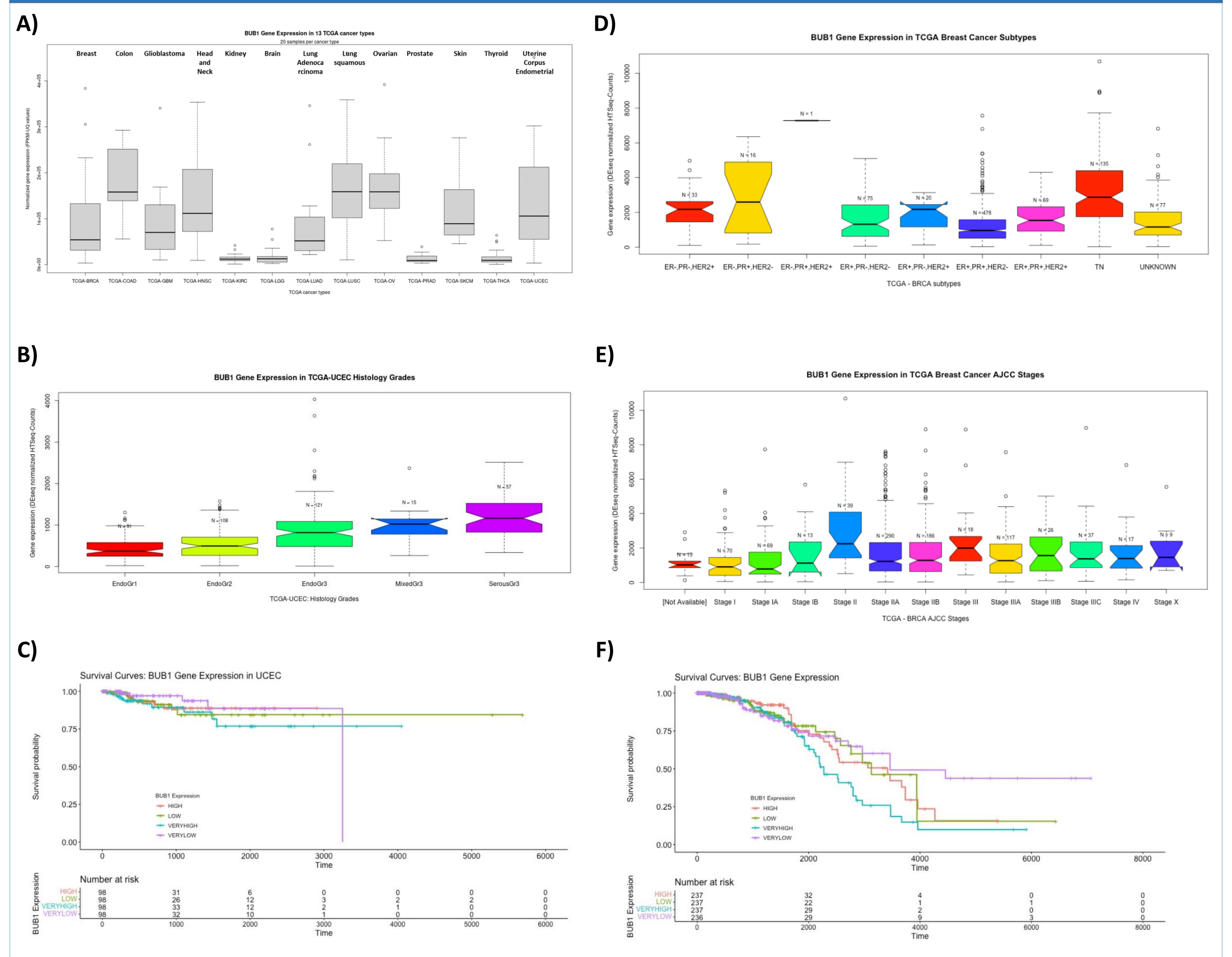
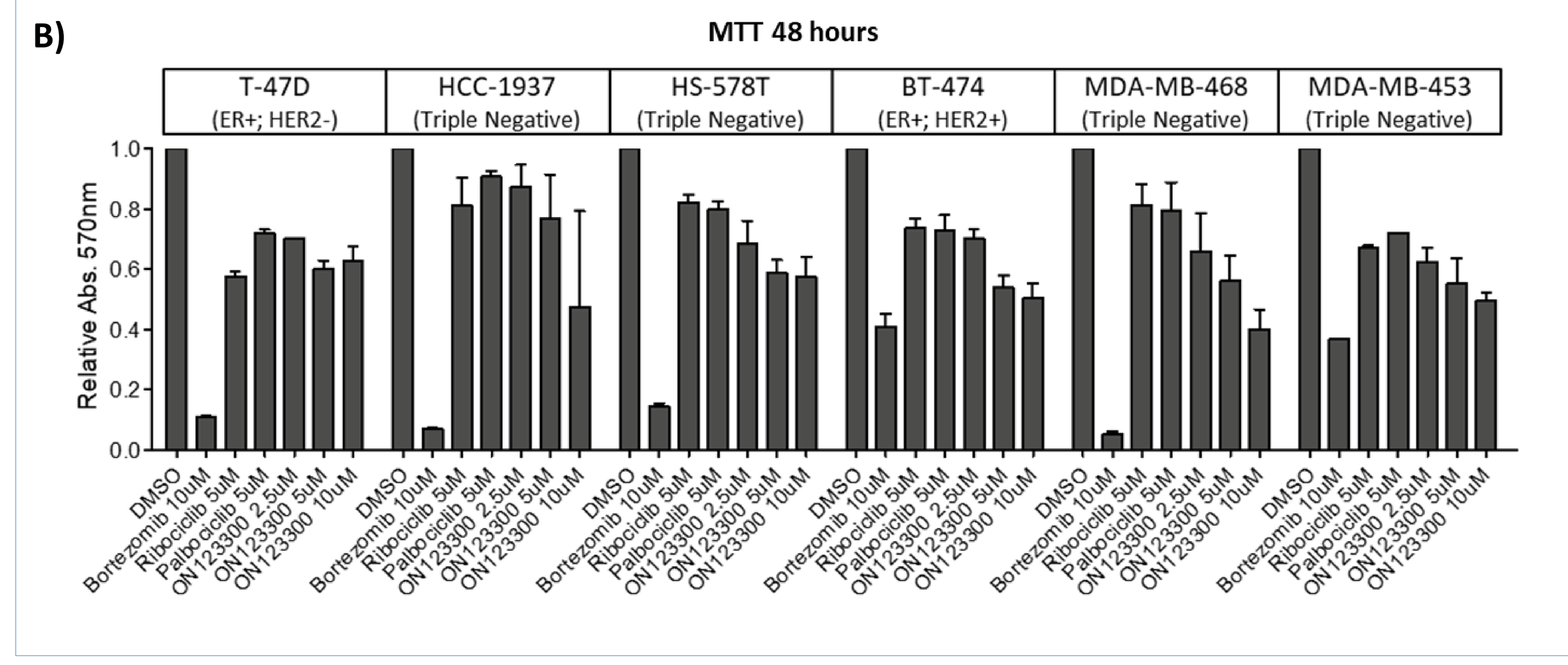


Figure 3. BUB1 expression levels in cancer patients based on the bioinformatics data from two human cancer databases (TCGA, CPTAC).
(A) BUB1 expression levels in different cancer types. **(B)** Higher BUB1 expression in Serous Grade 3 compared to endometrioid tumours. **(C)** VERY-HIGH BUB1 expression group has low survival probabilities in Uterine Corpus Endometrial Carcinomas (UCEC). **(D)** Triple Negative subtype has moderately higher median and overall BUB1 expression than other subtypes. **(E)** Higher BUB1 expression in AJCC Stage II. **(F)** VERY-HIGH BUB1 expression group has low survival probabilities in Breast Cancer.

Efficacy of ON123300 for the treatment of Breast Cancer

A)

Cell line	Cancer type	Treatment	48h - cell viability decrease	Known mutations
T-47D	HR+ HER2-	ON	60%	PIK3CA H1047R; TP53 L194F
BT-474	HR+ HER2+	ON	50%	MAPK1 H61Q; PIK3CA K111N; TP53 E285K
MDA-MB-453	TNBC	ON	50%	AR Q868H; CDH1 W638*; KRAS G13D; PIK3CA H1047R; PTEN Q307K
MDA-MB-468	TNBC	ON	65%	RB1c.265_2787del2523;TP53 R273H; PTEN c.253+1G>T
HCC-1937	TNBC	ON	20%	BRCA1 Q1756Pfs*74; TP53 R306*; RB1 del T738-775; PTEN deletion
Hs578T	TNBC	ON	40%	CDKN2A deletion; HRAS G12D; MT-CYB A125T; TP53 V157F
MDA-MB-231	TNBC	ON	60%	BRAF G464V; CDKN2A deletion; KRAS G13D; TP53 R280K;TERT c.1-124C>T



C)

Cell line	Cancer type	Treatment	BUB1	PARP cleavage	72h - cell viability decrease
MDA-MB-231	TNBC	ON	↘	Yes	75%
		ON+HCQ	↘	Yes	80%
		ON+SBI	↘	Yes	
		Abema	↘	Yes	
		Palbo	↘	No	
MDA-MB-468	TNBC	ON	↘	Yes	80%
		ON+HCQ	↘	Yes	80%
		ON+SBI	↘	Yes	90%
		Abema	-	No	50%
		Abema+HCQ	↘	Yes	45%
		Abema+SBI	↘	Yes	80%
		Palbo	-	No	10%
		Palbo+HCQ	↘	No	20%
		Palbo+SBI	↘	Yes	75%
HCC-1937	TNBC	ON	-	No	25%
		ON+HCQ	-	No	40%
		ON+SBI	-	No	50%
		Abema	-	No	10%
		Abema+HCQ	-	No	25%
		Abema+SBI	-	No	30%
		Palbo	-	No	15%
		Palbo+HCQ	-	No	25%
		Palbo+SBI	-	No	30%

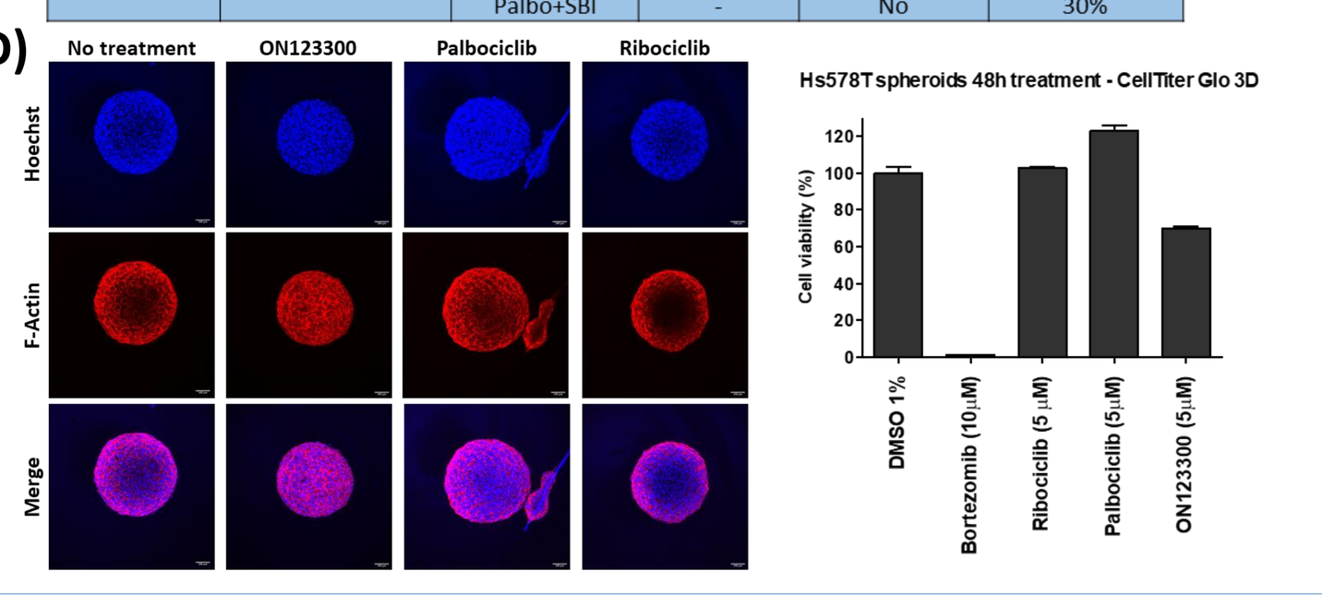


Figure 5. ON123300 effect on a panel of breast cancer cell lines.
(A) Description of the panel of breast cancer cell lines used in this study. Cell viability decrease is shown as measured with MTT assay after 48h treatment with ON123300 (5 mM). HR: hormone receptor; TNBC: triple negative breast cancer. **(B)** Cell viability measured using MTT assay with 48h treatment with the indicated compounds. Shown are percentage of viable cells relative to DMSO control as mean ±SEM from 3 independent experiments. **(C)** Effect of autophagy inhibitors in combination with CDK4/6 inhibitors on TNBC viability. **(D)** Representative images and quantification of the effect of the indicated compounds to the Hs578T spheroids viability by using the CellTiter-Glo® 3D Cell Viability Assay.

ON123300 induces both caspase-dependent and independent cell death

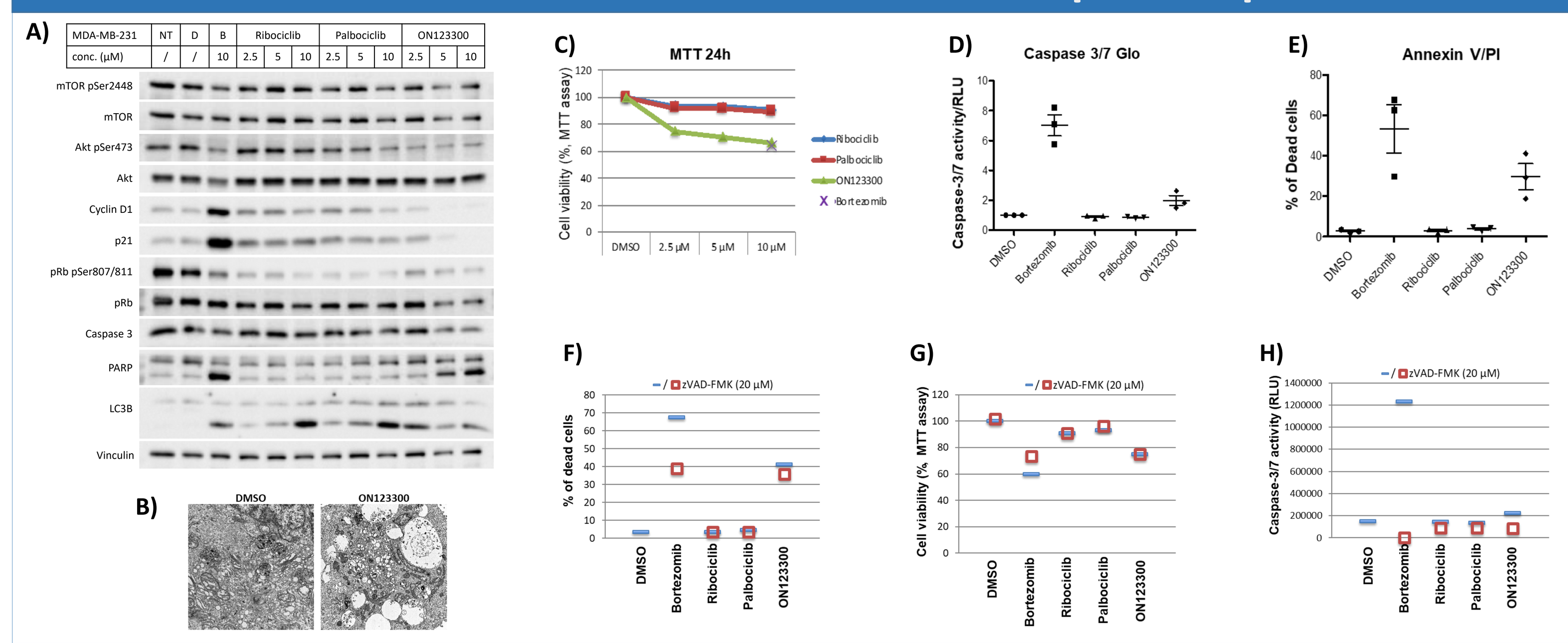
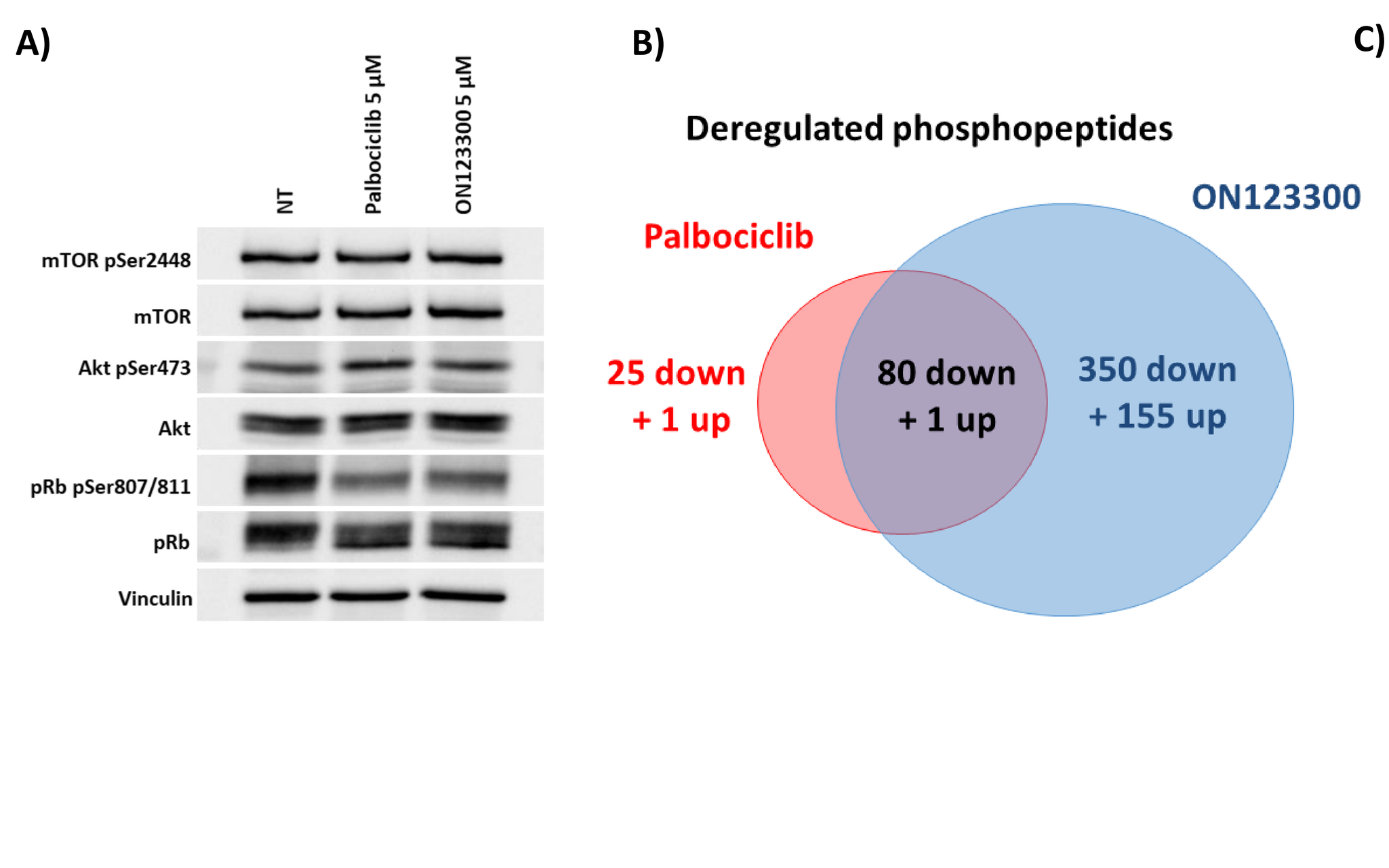


Figure 6. ON123330 induces both caspase-dependent and independent cell death in MDA-MB-231 cells.
MDA-MB-231 cells (TNBC) non treated (NT) or treated for 24 hours with DMSO (D), bortezomib (B) (10 µM), ribociclib (5 µM), palbociclib (5 µM), ON123300 (5 µM), and zVAD FMK (20 µM). **(A)** Western blot analysis of total cell lysates. **(B)** TEM imaging of MDA-MB-231 cells treated with DMSO or ON123300. **(C)** Cell viability measured using MTT assay. Shown are the percentages of viable cells relative to DMSO control from one out of two independent experiments. **(D)** Caspase 3/7 activity measured with Glo assay. Cells were treated with bortezomib, ribociclib, palbociclib or ON123300 relative to DMSO from 3 independent experiments. **(E)** Analysis of cell death using Annexin V/PI staining followed by FACS. Shown are the percentages of dead cells after treatment with bortezomib, ribociclib, palbociclib or ON123300 from 3 independent experiments. **(F)** Analysis of cell death using Annexin V/PI staining followed by FACS. **(G)** Cell viability measured using MTT assay, represented as percentages of viable cells relative to DMSO control. **(H)** Caspase 3/7 activity measured with Glo assay. Shown are the results for one out of two independent experiments.

Integrative Inferred Kinase Activity (INKA) analysis of MDA-MB-231 cells treated with ON123300 compared to palbociclib

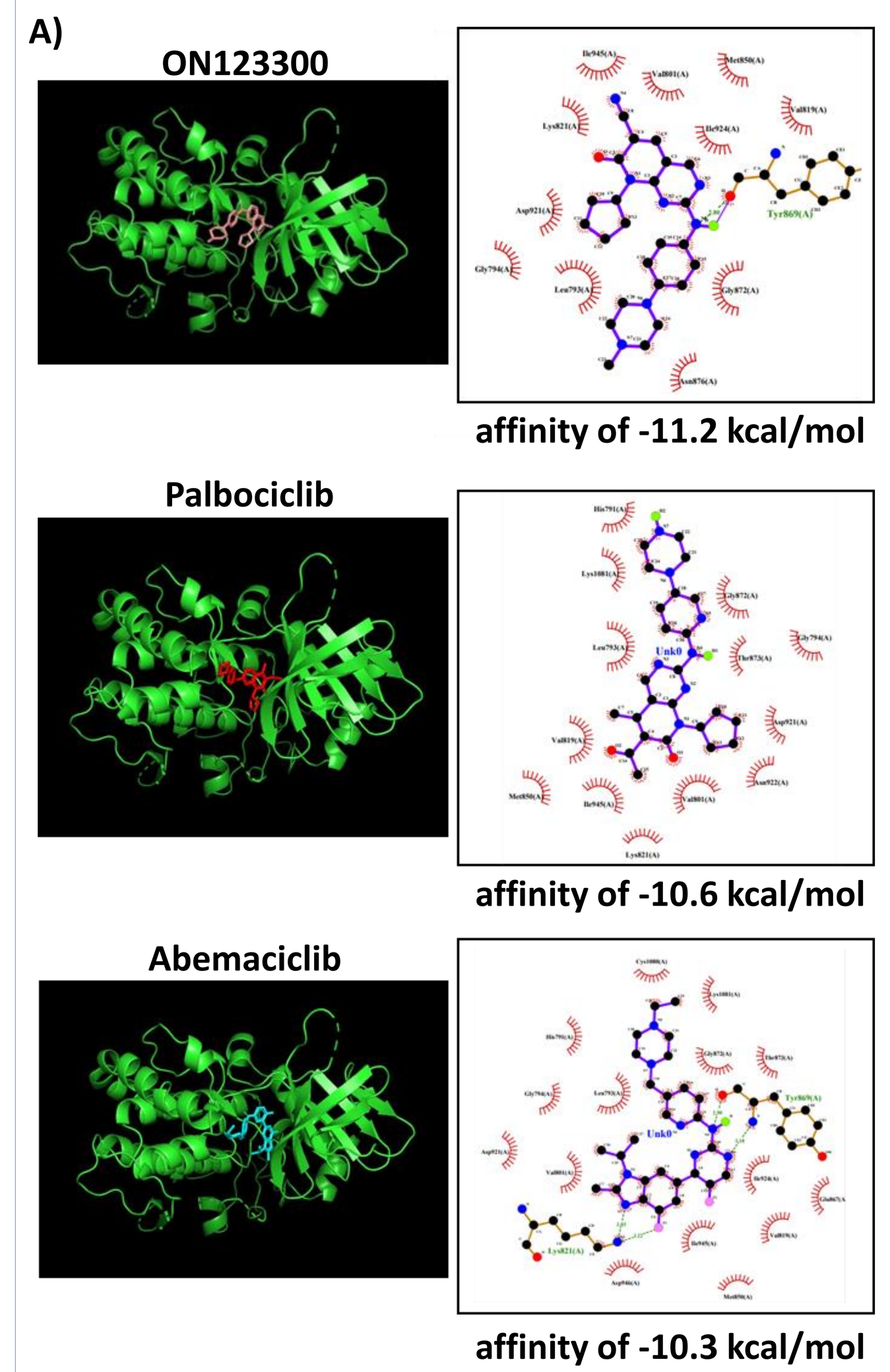


C)

Targets	Significance
CSNK1A1	Statistically significant both for ON123300 and Palbociclib treated cells
MAP3K11	
TESK1	
ATR	
PRKD1	Statistically significant only for ON123300 treated cells and between the two treatments
ULK1	
RAF1	
MAP2K2	
CAMK2D	
LYN	
PRKD2	
AKT2	
TLK1	
GTF2F1	
STK3	
CAMK1	
LATS1	
BRAF	
NUAK1	
HCK	
BUB1	
ARAF	
MAP2K5	
CDK16	
MAPK7	
CDK7	Statistically significant only for Palbociclib treated cells but not between treatments

Figure 2. Integrative data analysis pipeline for phosphoproteomic inference of active kinases changes of MDA-MB-231 cells treated for 2 hours with ON123300 compared to palbociclib.
(A) Western blot analysis of MDA-MB-231 cells lysates after 2h treatment with ON123300 or palbociclib, from a sample of the cells used for phosphoproteome analysis. Shown is the result for one sample out of three independent replicates. **(B)** Venn diagram representing the number of deregulated phosphopeptides in palbociclib-treated cells compared to ON123300-treated cells. **(C)** ON123300 and palbociclib unique targets, based on T-test statistic test ($p<0,05$).

Prediction of the binding-conformation of ON123300 to BUB1 binding site



B)

Residue	Restype	Interaction	ON	Abema	Palbo
821	LYS	Hydrogen bonds	#	#	
869	TYR	Hydrogen bonds	#	#	
869	TYR	Hydrogen bonds	#		
946	ASP	Hydrogen bonds	#		
791	HIS	Hydrophobic		#	#
793	LEU	Hydrophobic	#	#	#
794	GLY	Hydrophobic	#	#	#
801	VAL	Hydrophobic	#	#	#
819	VAL	Hydrophobic	#	#	#
821	LYS	Hydrophobic	#		#
850	MET	Hydrophobic	#	#	#
867	GLU	Hydrophobic		#	
872	GLY	Hydrophobic	#	#	#
873	THR	Hydrophobic	#	#	#
876	ASN	Hydrophobic	#		
921	ASP	Hydrophobic	#	#	#
922	ASN	Hydrophobic			#
924	ILE	Hydrophobic	#	#	
945	ILE	Hydrophobic	#	#	#
946	ASP	Hydrophobic		#	
1080	CYS	Hydrophobic		#	
1081	LYS	Hydrophobic		#	#

***793-801 and 821** are involved in the stabilization of the ATP molecule

C)

48h	1	2	3	4	5	6	7	8	9	10	11	12
DMSO	+	/	/	/	/	/	/	/	/	/	/	/
Abema	/	1 µM	/	/	/	/	/	/	/	/	/	/
Palbo	/	/	5 µM	/	/	/	/	/	/	/	/	/
Ribo	/	/	/	5 µM	/	/	/	/	/	/	/	/
SBI	/	/	/	/	10 µM	/	/	/	10 µM	10 µM	/	/
HCQ	/	/	/	/	/	15 µM	/	/	/	/	15 µM	15 µM
ON	/	/	/	/	/	/	5 µM	1 µM	5 µM	1 µM	5 µM	1 µM

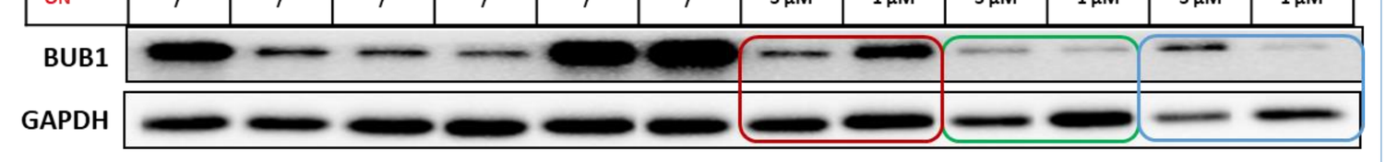


Figure 4. Molecular docking of CDK4/6 inhibitors - BUB1 interaction profile.
Prediction of **(A)** the binding-conformation and **(B)** binding sites of ON123300, palbociclib and abemaciclib to BUB1. **(C)** Western blot analysis of total cell lysates of MDA-MB-231 cells treated for 48 hours with CDK4/6 inhibitors or ON123300 in combination with autophagy inhibitors, hydroxychloroquine (HCQ) and SBI-0206965 (SBI).

Inhibition of autophagy sensitizes MDA-MB-231 cells to ON123300-induced apoptosis

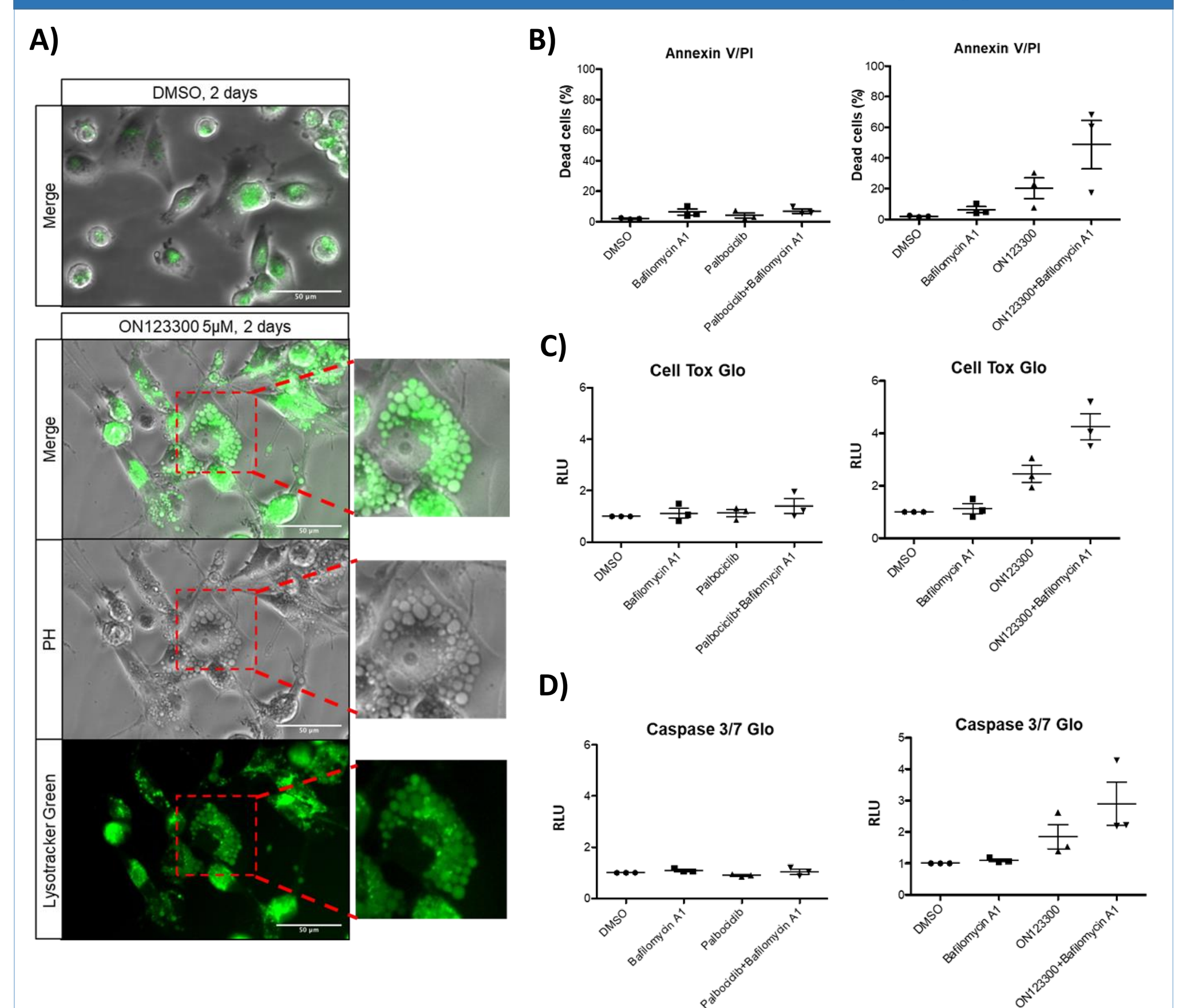


Figure 7. ON123300 induces cell death and vacuolization in MDA-MB-231 cells.
(A) Morphological changes of MDA-MB-231 cells after 2 days treatment with ON123300 (5 mM). Phase contrast (PH) microscopy images show formation of large vacuoles in the cytoplasm, that colocalize with Lysotracker Green staining. **(B)** Analysis of cell death using Annexin V/PI staining followed by FACS. Shown are the percentages of dead cells after treatment with bafilomycin A1, palbociclib or ON123300. **(C)** Dead cells were quantified using CytoTox-Glo Cytotoxicity Assay. Shown are mean RLU values relative to DMSO control. **(D)** Caspase 3/7 activity measured with Glo assay. Shown are relative luminescence unit (RLU) values relative to DMSO control.

Effect of ON123300 on PYMT mouse breast cancer cells

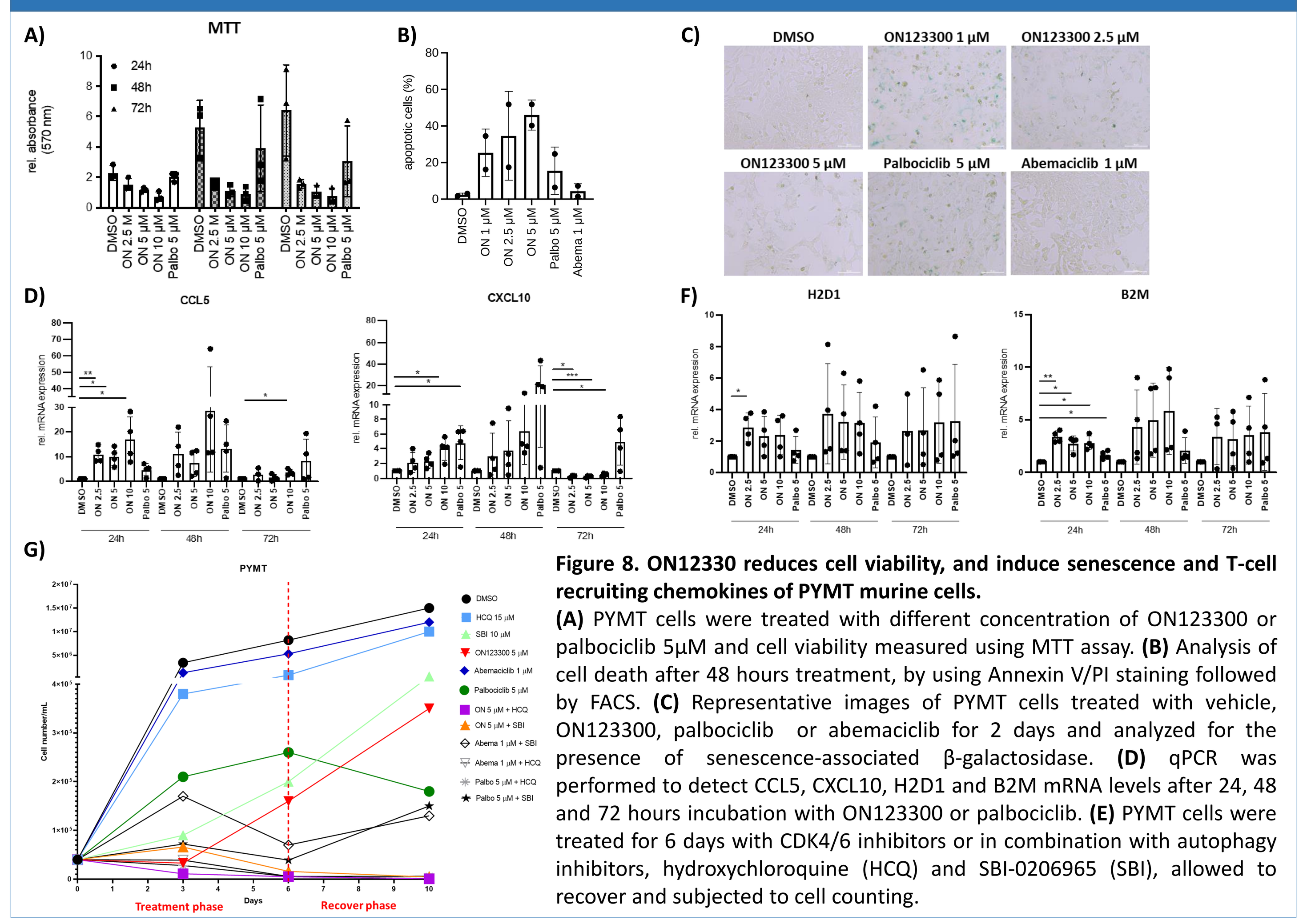


Figure 8. ON123300 reduces cell viability, and induce senescence and T-cell recruiting chemokines of PYMT murine cells.
(A) PYMT cells were treated with different concentration of ON123300 or palbociclib 5µM and cell viability measured using MTT assay. **(B)** Analysis of cell death after 48 hours treatment, by using Annexin V/PI staining followed by FACS. **(C)** Representative images of PYMT cells treated with vehicle, ON123300, palbociclib or abemaciclib for 2 days and analyzed for the presence of senescence-associated β-galactosidase. **(D)** qPCR was performed to detect CCL5, CXCL10, H2D1 and B2M mRNA levels after 24, 48 and 72 hours incubation with ON123300 or palbociclib. **(E)** PYMT cells were treated for 6 days with CDK4/6 inhibitors or in combination with autophagy inhibitors, hydroxychloroquine (HCQ) and SBI-0206965 (SBI), allowed to recover and subjected to cell counting.

Conclusions

- Narazaciclib (ON123300) treatment lead to BUB1 protein degradation, overexpression of which is associated with poor prognosis in TNBC.
- Combination of narazaciclib with autophagy inhibitors sensitizes several breast cancer cells to cell death.
- Narazaciclib treatment may promote antitumor immunity by influencing the expression of various immune modulators in the tumor cells which needs to be further validated in preclinical animal models; and ultimately in the clinic.

Contact

Petros Kechagioglou, PhD
University Medicine at the Johannes Gutenberg University in Mainz
Email: pkechagi@uni-mainz.de

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