Narazaciclib's differential targets and kinase inhibitory activity compared to the approved CDK4/6 inhibitors contribute to the enhanced inhibition of tumor growth in preclinical models

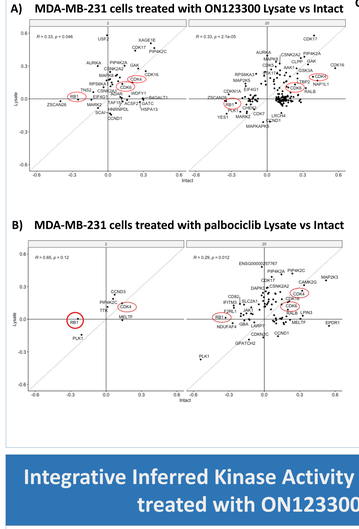
Petros Kechagioglou[1], Debomita Chakraborty[1], Camille Dupont[1], Hajime Yurugi[1], Ute Distler[2], Stefan Tenzer[2], Alexey Chernobrovkin[3], Kristina Riegel[1], Julianne Mooz[1], Mahil Lamber[4], Volker Dötsch[4], Stephen Cosenza[5], Steven M Fruchtman[5] and Krishnaraj Rajalingam[1]

[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

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 and in endometrial cancers in combination with letrozole (NCT05705505).
- Despite clinical benefit, safety concerns such as neutropenia and diarrhea, and disease progression, raises a critical need to identify novel therapeutic strategies.
- **Aim**: Explore the activity of narazaciclib and its metabolite ON1232580 in comparisons to the FDA approved CDK4/6i and identify additional targets engaged by narazaciclib.

Identification of cellular targets based on the Cellular Thermal Shift Assay (CETSA) profiling of MDA-MB-231 cells treated with ON123300 compared to palbociclib (CDK4/6 inhibitor)



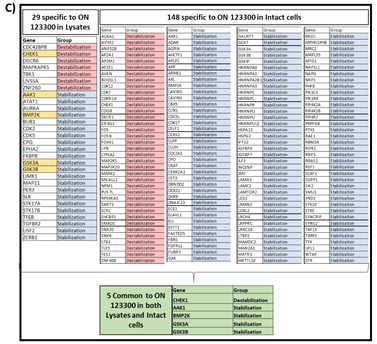


Figure 1. A mass spectrometry-based cellular Thermal Shift Assay (CETSA-MS) profiling of MDA-MB-231.
Both lysates and intact cells were treated with **(A)** ON123300 (2 μM and 20 μM) or **(B)** palbociclib (2 μM and 20 μM). **(C)** Table with list of the potential unique ON123300 targets compared to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

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

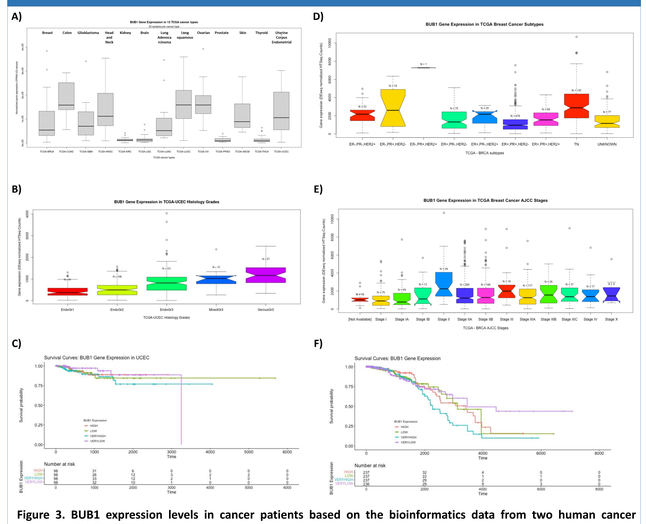
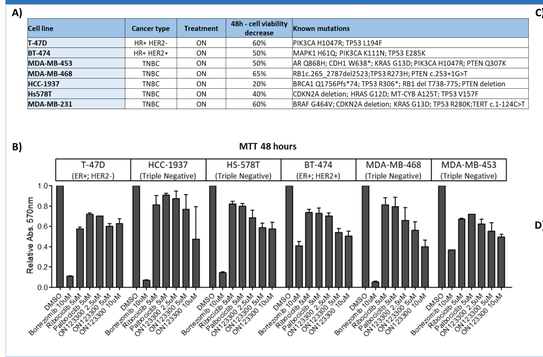


Figure 3. BUB1 expression levels in cancer patients based on the bioinformatics data from two human cancer databases (TCGA, CPTAC).
(A) BUB1 expression levels in different cancer types. **(B)** Higher BUB1 expression in Serous Grade 3 compared to endometrioid tumours. **(C)** VERY-HIGH BUB1 expression 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 QB68H; CDH1 W638*; KRAS G13D; PIK3CA H1047R; PTEN Q307K
MDA-MB-468	TNBC	ON	65%	RB1c.265_278del(p.S723_R732fs); 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



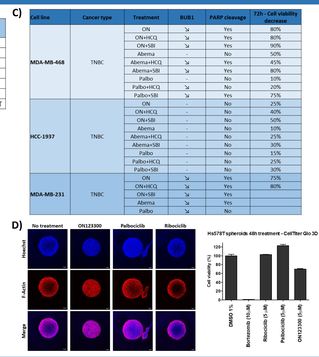
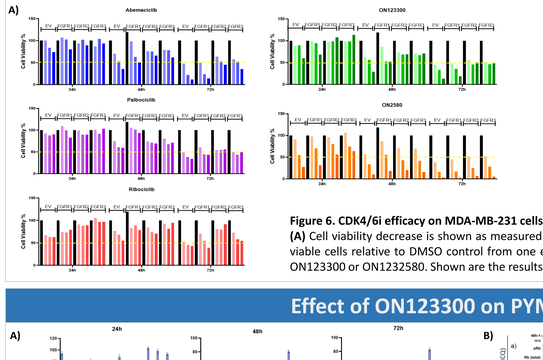


Figure 5. ON123300 effect on a panel of breast cancer cell lines.
(A) Description of the panel of breast cancer cell lines used in this study. Cell viability decrease is shown as measured with MTT assay after 48h treatment with ON123300 (5 μM). HR: hormone receptor; TNBC: triple negative breast cancer. **(B)** Cell viability measured using MTT assay after 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.

FGFR overexpressing MDA-MB-231 (TNBC) cells retain sensitivity towards ON123300 and ON1232580



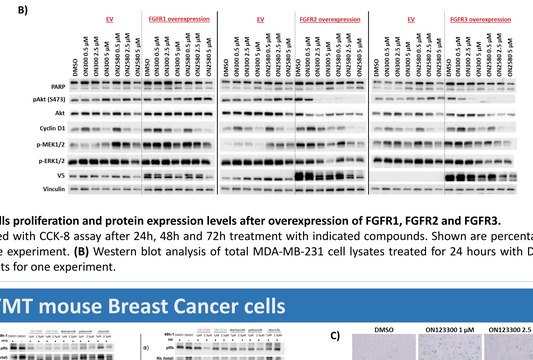
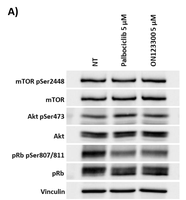
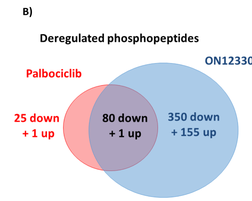


Figure 6. CDK4/6i efficacy on MDA-MB-231 cells proliferation and protein expression levels after overexpression of FGFR1, FGFR2 and FGFR3.
(A) Cell viability decrease is shown as measured with CCK-8 assay after 24h, 48h and 72h treatment with indicated compounds. Shown are percentage of viable cells relative to DMSO control from one experiment. **(B)** Western blot analysis of total MDA-MB-231 cell lysates treated for 24 hours with DMSO, ON123300 or ON1232580. Shown are the results for one experiment.

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





C)

Targets	Significance
CSNK1A1 MAP3K11 TESK1 ATR	Statistically significant both for ON123300 and Palbociclib treated cells
PRKD1 ULK1 RAF1 MAP2K2 CAMK2D LYN PRKD2 AKT2 TLK1 GTF2F1 STK3 CAMK1 LATS1 BRAF NUAK1 HCK BUB1 ARAF MAP2K5 CDK16 MAPK7	Statistically significant only for ON123300 treated cells and between the two treatments
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 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 ($p<0,05$). **(D)** 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).

ON123300 and ON1232580 (metabolite) are more potent against cyclins compared to abemaciclib and palbociclib in Breast Cancer cell lines MCF7, T47D (HR+/HER2-) and Ovarian Adenocarcinoma OvCar3

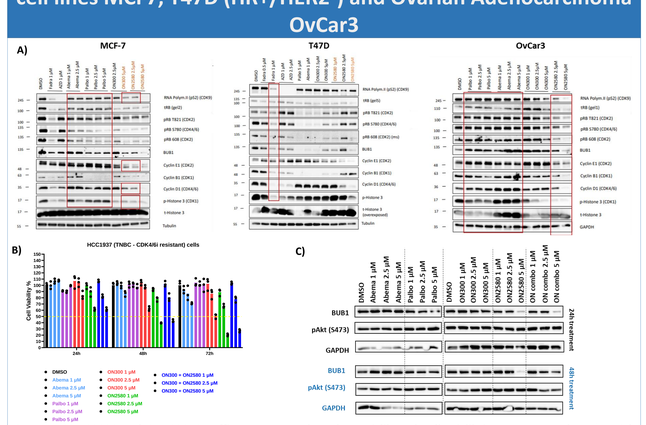


Figure 4. ON123300 and ON1232580 efficacy compared to abemaciclib and palbociclib in MCF7, T47D (HR+/HER2-), HCC1937 (TNBC – CDK4/6i resistant) and Ovarian Adenocarcinoma OvCar3 cells.
(A) Western blot analysis of cell lysates treated for 24 hours with indicated compounds. Shown are the results for one experiment. Replicates and quantification is pending. Different concentrations (0.5 μM, 1 μM, 2.5 μM and 5 μM) were examined for all the compounds – data not shown here. **(B)** Cell viability decrease is shown as measured with CCK-8 assay after 24, 48 and 72 hours treatment with vehicle, ON123300, ON1232580, palbociclib, ribociclib or abemaciclib. Shown are percentage of viable cells relative to DMSO control for one experiment. **(C)** Western blot analysis of total cell lysates of HCC1937 cells treated for 24 and 48 hours with the indicated compounds.

Effect of ON123300 on PYMT mouse Breast Cancer cells

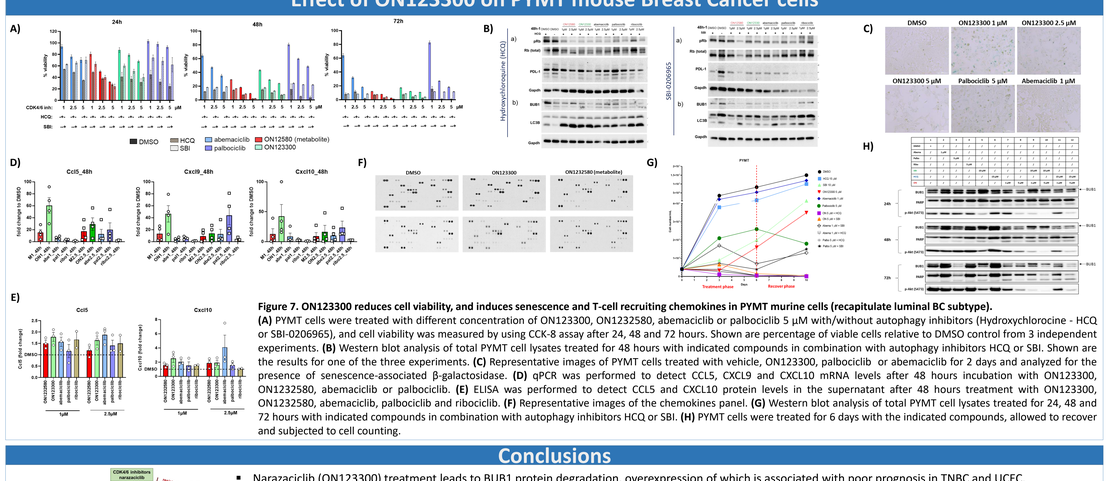
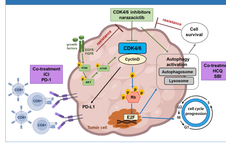


Figure 7. ON123300 reduces cell viability, and induces senescence and T-cell recruiting chemokines in PYMT murine cells (recapitulate luminal BC subtype).
(A) PYMT cells were treated with different concentration of ON123300, ON1232580, abemaciclib or palbociclib 5 μM with/without autophagy inhibitors (Hydroxychloroxine - HCQ or SBI-0206965), and cell viability was measured by using CCK-8 assay after 24, 48 and 72 hours. Shown are percentage of viable cells relative to DMSO control from 3 independent experiments. **(B)** Western blot analysis of total PYMT cell lysates treated for 48 hours with indicated compounds in combination with autophagy inhibitors HCQ or SBI. Shown are the results for one of the three experiments. **(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, CXCL9 and CXCL10 mRNA levels after 48 hours incubation with ON123300, ON1232580, abemaciclib or palbociclib. **(E)** ELISA was performed to detect CCL5 and CXCL10 protein levels in the supernatant after 48 hours treatment with ON123300, ON1232580, abemaciclib, palbociclib and ribociclib. **(F)** Representative images of the chemokines panel. **(G)** Western blot analysis of total PYMT cell lysates treated for 24, 48 and 72 hours with indicated compounds in combination with autophagy inhibitors HCQ or SBI. **(H)** PYMT cells were treated for 6 days with the indicated compounds, allowed to recover and subjected to cell counting.

Conclusions



- Narazaciclib (ON123300) treatment leads to BUB1 protein degradation, overexpression of which is associated with poor prognosis in TNBC and UCEC.
- Narazaciclib and its metabolite ON1232580 exhibit the most potent cytotoxic effect among other CDK4/6 inhibitors, especially in PYMT mouse cells.
- FGFR overexpressing cells still retain sensitivity towards ON123300 and ON1232580.
- Inhibition of autophagy sensitizes cells to both ON123300 and ON1232580, and induce irreversible proliferation inhibition, providing a novel therapeutic approach.
- Narazaciclib and its metabolite treatment may promote antitumor immunity by influencing the expression of various immune modulators in the tumor cells which will be 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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Acknowledgements

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