Abstract # 3029

Dual targeting of ARK5 and CDK4 pathways with ON123300 as a therapeutic strategy for colorectal carcinoma

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ONCONOVA THERAPEUTICS

Abstract

Introduction:

This study describes the development of a novel dual specificity kinase inhibitor, ON123300, which exhibits potent activity against colorectal cancers both *in vitro* and *in vivo*. While over-expression of Cyclin D1 has been found to closely correlate with the proliferation rate of the tumor cells, metastatic colorectal cancers over express ARK5 , a member of the AMPK family which has been shown to mediate AKT activation. In this study, we show that ON123300 , which inhibits both CDK4/6 and ARK5, is a potent inducer of apoptosis of colorectal cancer cells when compared to palbociclib, a highly selective inhibitor of CDK4/6 that does not target ARK5.

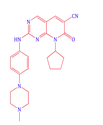
Results and Conclusions:

We examined the effects of palbociclib and ON123300 on cell progression, modulation of the RB and PI3K/AKT pathways, and induction of apoptosis in multiple colorectal cancer cell lines. Comparative kinase inhibition assays showed that while palbociclib and ON123300 exhibited equivalent inhibition against CDK4/CDK6, ARK5 activity was inhibited only by ON123300. When DLD1 and SW480 cells were incubated with increasing concentrations of palbociclib or ON123300, both compounds were equally efficient in their ability to inhibit phosphorylation of all three members of the Rb family of proteins. However, when the phosphorylation status of proteins associated with the PI3K/AKT pathway was measured by western blot analysis, ON123300 showed concentration-dependent inhibition of 4EBP1 and S6RB phosphorylation while palbociclib had little or no effect on the phosphorylation of these proteins. Cells treated with palbociclib rapidly accumulated in the G0/G1 stage of the cell cycle with increasing drug concentrations. Although cells treated with ON123300 also arrested in the G0/G1 phase at lower concentrations (0.1 -0.5 µM), with increasing concentrations of drug, there was an accumulation of cells with sub-G1 DNA content, suggesting induction of apoptosis. ON123300-treated cells showed cleavage of PARP and Caspases 3,7,9 as well as inhibition of FOX01 phosphorylation, which was not observed in cells treated with palbociclib. Since ARK5 belongs to the AMPK family of kinases, we next examined the effects of ON123300-mediated ARK5 inhibition on metabolic changes of tumor cells that overexpress this gene. Treatment of SW-480 colorectal cancer cells with ON123300 resulted in an increase in glucose uptake, profound inhibition of glutamine uptake and reduced ATP production. A detailed metabolomic study revealed significant alterations in the levels of metabolites associated with glutamine metabolism. Nude mouse xenograft assays using Colo-205 cells revealed strong inhibition of tumor growth following 100mg/kg of ON123300 given QD or QOD, with little evidence of toxicity as measured by change in body weight. Thus, dual inhibition of ARK5 and CDK4 pathways could be an effective therapeutic strategy for the treatment of colorectal cancers.

Kinase inhibition profile for ON123300

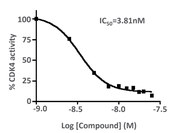
A

Structure of ON123300



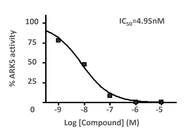
B

CDK4 Kinase Assay



C

ARK5 Kinase Assay



D

GI_{50} Values Against a Panel of Human Tumor Cell lines

CELL LINE	Tumor Type	GI50 (µM) ON123300
DLD-1	Colorectal	0.1
SW480	Colorectal	0.1
MIA-PaCa-2	Pancreatic	0.25
HCT-15	Colon (MDR elevated)	0.4
COLO-205	Colon	0.2
SNU-5	Gastric	0.2
SNU-398	Gastric	0.5
MDA-MB-231	Breast (TN)	0.2
MDA-MB-157	Breast (TN)	0.25
BT474	ErbB2 + Breast	0.25
SK-BR-3	ErbB2 + Breast	0.6
MCF-7	ER+ Breast	0.15
BT20	Breast	0.1

E

Kinase inhibition profile of ON123300

Kinase	ON123300 IC_{50}(nM)	Palbociclib IC_{50}(nM)
ARK5	4.95	>5000
CDK4/Cyclin D1	3.87	5.36
CDK6/Cyclin D1	9.82	3.76
FGFR1	26.0	>10,000
PDGFRβ	26.0	>10,000
PI3K-δ	144.0	>10,000

F

PI3K Kinase inhibition profile of ON123300

Kinase	ON123300 IC_{50}(nM)	LY294002 IC_{50}(nM)
PI3Kalpha	1060	134
PI3Kbeta	759	533
PI3Kdelta	144	267
PI3Kgamma	1119	1680

(A) Structure of ON123300 (B) Cdk4 kinase inhibition by ON 123300. (C) Inhibition of PI3K-δ by ON123300. (D) Human tumor cell killing activity of ON123300. Note the high degree of activity against Colorectal cancer lines compared to other tumor types. (E) kinase inhibition profile of ON123300 (F) PI3Kinase inhibition profile of ON 123300.

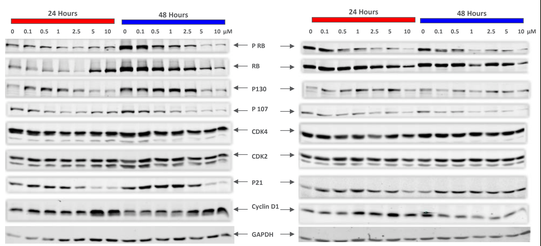
ON123300 and Palbociclib inhibit the phosphorylation of CDK4 targets

A

ON123300

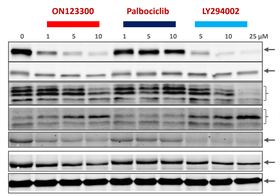
B

Palbociclib



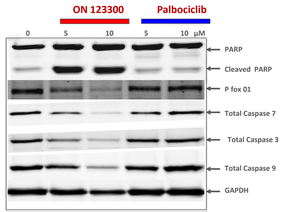
SW480 cells were treated with the specified concentration of ON123300 or Palbociclib. 30-50 µg of clarified protein from these lysates was resolved on SDS page and processed for western blot analysis. Treatment of ON123300 and Palbociclib results in inhibition of CDK4/6-mediated phosphorylation of Rb family of proteins and their downstream targets.

Inhibition of PI3K-mediated Signaling by ON123300.



SW480 cells were treated with increasing concentrations of ON123300 or Palbociclib for 24 hours. Whole cell extracts were subjected to western blot analysis using antibodies directed against the indicated proteins. GAPDH is shown as a loading control. Treatment of ON123300 inhibits mTORC and its downstream targets which are not inhibited by Palbociclib.

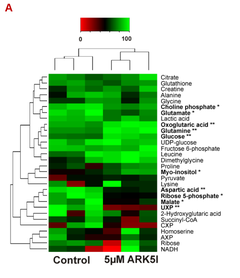
SW480 cells Treated with ON123300 undergo Apoptosis



Sw480 cells were treated with the indicated concentrations of ON123300 or Palbociclib. 30-50 µg of clarified cell extracts were resolved by SDS page and the expression levels of the indicated proteins determined by western blot analysis. Treatment with ON123300 but not Palbociclib leads to activation of PARP and caspases 3,7,and 9.

Metabolite heatmap for SW480 cells treated with ON123300

A



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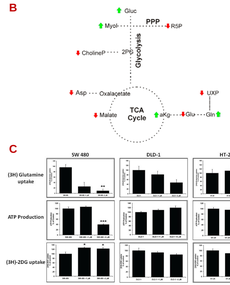


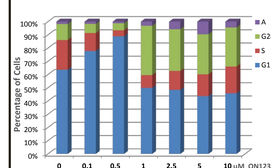
C

(A) Heatmap for SW480 cells treated with 5µM ON123300 for 12 hrs. Significantly altered metabolites are indicated in bold. Standard T test using Excel has been used to calculate the p-values. (* <0.05, **<0.01, and ***<0.001) (B) Pathway generated from HSQC data; the indicated metabolites are significantly altered due to ARK5 inhibitor treatment (C) Treatment of ON123300 in ARK5 expressing cells SW480 , DLD1 leads to reduced Glutamine uptake and ATP production.

Cell cycle analysis by PI staining and flow cytometry.

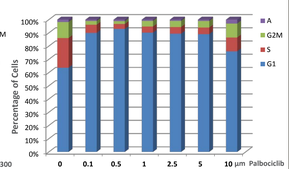
A

FACS analysis of ON123300



B

FACS analysis of Palbociclib



(A) Percentage of Sw480 Cells in cell cycle after treatment with ON123300

(B) Percentage of Sw480 Cells in cell cycle after treatment with Palbociclib

Effect of ON123300 on the growth of Colon 205 tumor cells in a mouse xenograft model

A

In vivo Toxicity studies

GROUP	Formulation	ROUTE	Schedule	Results/ Survival
50 mg/kg	HCL-H2O	IP	Single	5/5
100 mg/kg	HCL-H2O	IP	Single	5/5
100 mg/kg	HCL-H2O	IP	QDX5	3/3

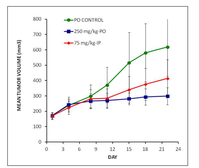
B

Plasma levels of ON123300

GROUP	DOSE	ROUTE	AUC (µg/ml/hr)	Drug concentration µM/ml/hr
123300-HCL	50 mg/kg	IP	7.18	16.710
123300-HCL	50 mg/kg	PO	2.78	6.470

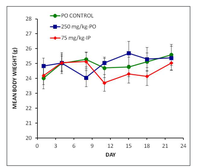
C

Mean Tumor Volumes



D

Mean Body Weight



(A) Female CD-1 mice were injected with 50 or 100mg/kg of ON123300 as a single dose or for 5 consecutive days. The mice were observed daily for survival and signs of toxicosis. No noticeable toxicity was observed in mice after treatment. (B) Female CD-1 mice were injected, in duplicate, with ON 123300 formulated in sterile H2O as an HCL salt. Plasma was obtained and ON123300 levels were determined by LC-MS/MS. (C) Colo205 cells were injected into nude mice and allowed to grow to approximately 100mm³ in size and treated daily with either vehicle or ON123300. Representative image of tumors in control and ON123300-treated mouse cohorts.. (D) Mean Body Weights

CONCLUSION

1. Palbociclib and ON123300 exhibited equivalent potency in the inhibition of CDK4/CDK6 kinase activities while ARK5 activity was inhibited only by ON123300.

2. Palbociclib and ON123300 were equally efficient in their ability to inhibit phosphorylation of all three members of the Rb family of proteins.

3. ON123300 showed concentration-dependent inhibition of 4EBP1 and S6RB phosphorylation while Palbociclib had little or no effect on the phosphorylation of these proteins.

4. ON123300-treated cells showed cleavage of PARP and CASPASES (3,7,9) as well as inhibition of FOX01 phosphorylation, which was not observed in cells treated with Palbociclib.

5. Treatment of SW480 colorectal cancer cells with ON123300 resulted in an increase in glucose uptake, profound inhibition of glutamine uptake and reduced ATP production.

6. A detailed metabolomic study revealed significant alterations in the levels of metabolites associated with glutamine metabolism.

7. ON123300 effectively inhibited the growth of Colo205 tumor cells in a xenograft model, suggesting that dual inhibition of ARK5 and CDK4 pathways could be an effective therapeutic strategy for the treatment of colorectal cancers.

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