Discovery of BTX-9341, a bifunctional degrader of CDK4 and CDK6 for HR+/HER2- breast cancer

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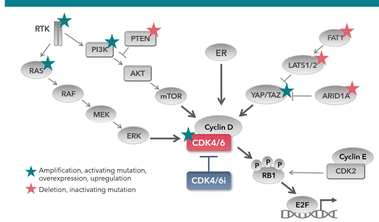
BACKGROUND



CDK4 and CDK6 are kinases which regulate cell cycle progression through the phosphorylation of retinoblastoma protein (RB) which releases the transcription factor E2F, driving the expression of cell cycle promoting genes. CDK4/6 are clinically validated targets in ER+/HER2- breast cancer, with multiple CDK4/6 inhibitors (CDK4/6i) approved for use in this indication, but resistance remains an issue with >20% of patients exhibiting intrinsic resistance and up to 70% of patients developing acquired resistance within 3 years.[1] Many resistance mechanisms converge on the upregulation of CDK6.[2-5] To address this we sought to generate CDK4/6 bifunctional degraders.

METHODS

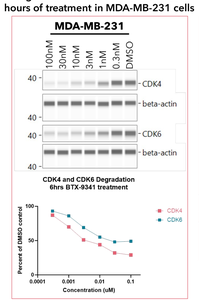
- PRODEGY platform was utilized to develop a series of Cereblon (CRBN) mediated CDK4/6 bifunctional degraders including development candidate BTX-9341.
- Knockout cell lines were generated by nucleofection of Cas9-gRNA complexes.
- Target degradation was analyzed by immunoblots of protein lysates from cells treated with BTX-9341 for 6 hours or as indicated.
- Phosphorylated Rb was analyzed by in cell western after 24 hours of treatment or by immunoblot where indicated.
- Cell cycle analysis was performed after 24 hours of treatment by flow cytometry following propidium iodide staining.
- E2F target gene expressed was analyzed by qPCR and immunoblot.
- Cell proliferation was measured by CellTiter-Glo 2.0 assay (Promega) after a 10-day colony formation assay.
- Vehicle, CDK4/6 inhibitor(s), and BTX-9341 were dosed orally in BALB/c nude mice xenograft subcutaneous models.

RESULTS

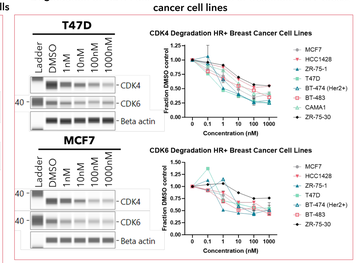
- BTX-9341 is a potent, CRBN and proteasome dependent degrader of CDK4 and CDK6 in multiple breast cancer cell lines. CDK4/6 degradation is rapid and sustained after compound washout.
- Kinome profiling indicates BTX-9341 is more selective than the CDK4/6i palbociclib at 100 nM, and proteomics indicates minimal off-target degradation.
- BTX-9341 inhibits downstream signaling, including :
  - Rb phosphorylation in breast cancer cells with pRb IC_{50}s below 50 nM.
  - Cell cycle progression, with cell cycle arrest at low nanomolar concentrations.
  - Downregulation of E2F target genes at the mRNA and protein level which is sustained over a period of 72 hours while CDK4/6 inhibitors show recovery of target gene expression.
  - Inhibition of proliferation, with colony formation assay IC_{50}s in the low nanomolar range. BTX-9341 has enhance potency compared to CDK4/6i which is dependent on CRBN.
- BTX-9341 exhibits synergy with the SERD fulvestrant in a colony formation assay to assess proliferation inhibition.
- BTX-9341 retains potency in a CDK4/6i resistant cell line and exhibits enhanced synergy with fulvestrant in this resistant cell line.
- BTX-9341 exhibits good tumor exposure when dosed orally, and induces a dose-dependent reduction in CDK4, CDK6, and pRb levels in MCF7 xenograft tumors. In this model, BTX-9341 exhibits dose dependent tumor growth inhibition and tumor regression at higher doses that was well correlated with CDK4, CDK6 and pRb downregulation.
- BTX-9341 also inhibits tumor growth in several other HR+/HER2- xenograft models

BTX-9341 exhibits rapid, potent and sustained CDK4 and CDK6 degradation

Degradation of CDK4 and CDK6 at 6 hours of treatment in MDA-MB-231 cells

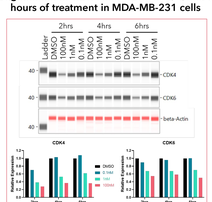


Degradation of CDK4 and CDK6 at 6 hours of treatment HR+ breast cancer cell lines

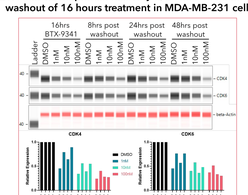


Representative Simple Western™ blots for two HR+ cells lines on the left. Quantified immunoblots for all tested HR+ cell lines on the right. CAMA1 cells do not express CDK6. BT-474 cell line is HER2+, all other cell lines are HER2-.

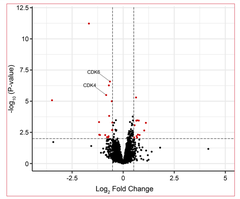
Degradation of CDK4 and CDK6 at 2, 4, and 6 hours of treatment in MDA-MB-231 cells



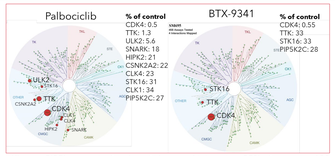
CDK4 and CDK6 protein recovery 8, 24 and 48 hours after washout of 16 hours treatment in MDA-MB-231 cells



BTX-9341 exhibits selective binding and degradation



TMT mass spectrometry performed on samples generated in triplicate from MDA-MB-231 cells treated with 100 nM of BTX-9341 or DMSO for 6 hours. Volcano plot indicating the fold change comparing BTX-9341 treated and DMSO treated cells. CDK4 and CDK6 are labeled as some of the few proteins which were significantly altered after BTX-9341 treatment.



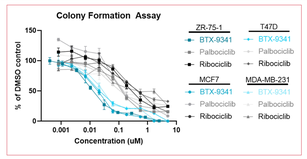
Kinome S(35) score: the fraction of kinases with less than 35% of control at 100nM among the 468 kinases tested with the KINOMEscan™ platform from Eurofins.

Compound	Kinome S(35) score @100nM
BTX-9341	0.01
Palbociclib	0.027

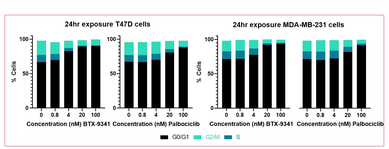
Compound	CDK6 Kd
BTX-9341	31 nM
Palbociclib	5.1 nM

BTX-9341 inhibits RB phosphorylation, cell cycle progression and cell proliferation alone and synergistically with fulvestrant

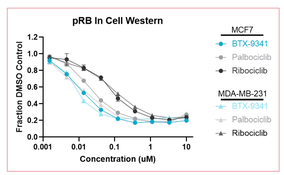
BTX-9341 inhibits cell proliferation



BTX-9341 induces cell cycle arrest

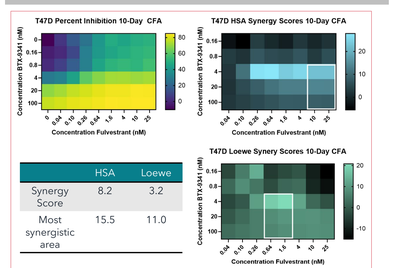


BTX-9341 inhibits Rb phosphorylation



BTX-9341 inhibits Rb phosphorylation after 24 hours of treatment. Data generated by in cell western for total RB and phosphorylated Rb (pSer807/811) taken as a ratio and normalized to DMSO treated control.

BTX-9341 exhibits synergy with fulvestrant in T47D cells

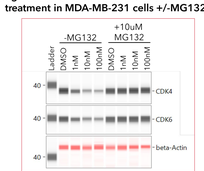


	HSA	Loewe
Synergy Score	8.2	3.2
Most synergistic area	15.5	11.0

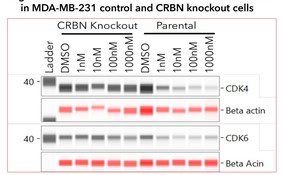
BTX-9341 potently inhibits downstream signaling and cell proliferation *in vitro* in HR+/HER2- BC cells and CDK4/6i resistant cells

BTX-9341 activity is dependent on the proteasome and Cereblon

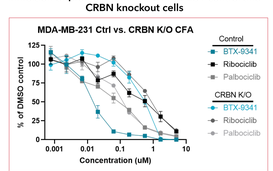
Degradation of CDK4 and CDK6 at 6 hours of treatment in MDA-MB-231 cells +/-MG132



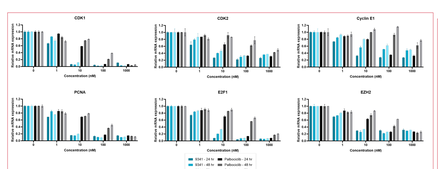
Degradation of CDK4 and CDK6 at 6 hours of treatment in MDA-MB-231 control and CRBN knockout cells



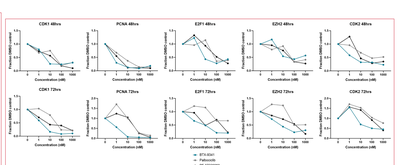
Inhibition of proliferation in MDA-MB-231 control and CRBN knockout cells



BTX-9341 downregulates E2F target genes at the mRNA and protein level

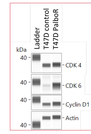


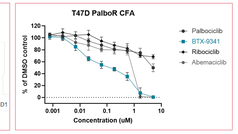
BTX-9341 inhibits E2F target gene mRNA expression in T47D cells. Suppression of gene expression is sustained through 72 hours, while recovery of gene expression is seen starting at 48 hours after palbociclib treatment

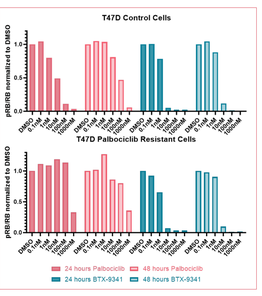


BTX-9341 inhibits E2F target gene expression at the protein level in T47D cells. Suppression of protein expression is deepened at 72 hours with BTX-9341 treatment, while recovery of protein expression is seen with some concentrations of palbociclib and PF-07220060

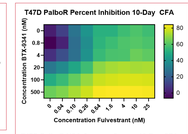
BTX-9341 inhibits cell proliferation, Rb phosphorylation and synergizes with fulvestrant in a palbociclib resistant cell line







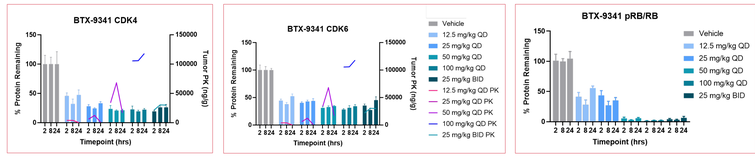
Inhibition of RB phosphorylation over time measured by immunoblot after treatment of T47D control and palbociclib resistant cells with BTX-9341 or palbociclib.



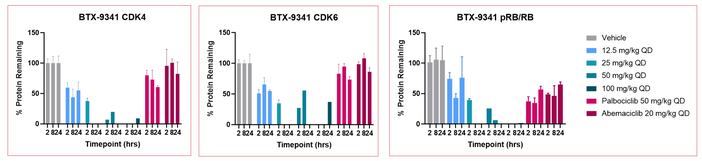
	HSA	Loewe
Synergy Score	10.7	7.4
Most synergistic area	21.5	19.0

BTX-9341 induces tumor regression in MCF7 xenograft model

BTX-9341 degrades CDK4, and CDK6, AND inhibits pRB in MCF7subcutaneous tumors

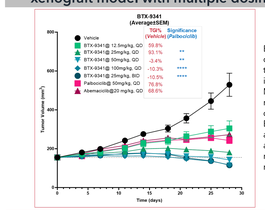


BTX-9341 exhibits dose-dependent target degradation of CDK4 and CDK6 which correlates with a dose-dependent decrease in phosphorylated RB relative to total RB that is rapid and sustained.

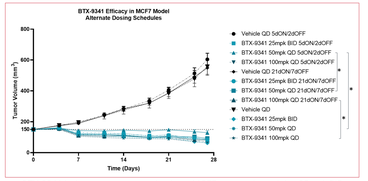


BTX-9341 exhibits dose-dependent target degradation of CDK4 and CDK6 which correlates with a dose-dependent decrease in phosphorylated RB relative to total RB in and MCF7 xenograft efficacy model. Decreases in pRB more significant than CDK4/6i at dose levels higher than 25mpk.

BTX-9341 induces tumor regression in an MCF7 xenograft model with multiple dosing schedules



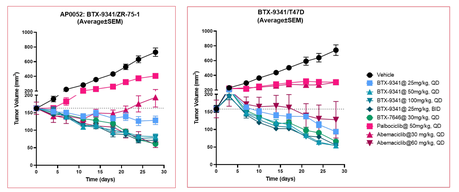
BTX-9341 exhibits dose-dependent tumor growth inhibition in an MCF7 xenograft model. BTX-9341 dosed at 25mpk BID, 50 mpk QD and 100 mpk QD all cause tumor regression in this model.



BTX-9341 induces tumor regression in an MCF7 xenograft model at 25mpk BID, 50mpk QD and 100 mpk QD in a 5 day on, 2 day off dosing schedule and a 21 day on 7 day off dosing schedule with minor but significant differences in potency compared to a full 28-day dosing for dose levels except 25 mpk BID.

BTX-9341 inhibits RB phosphorylation, cell cycle progression and cell proliferation alone and synergistically with fulvestrant

BTX-9341 inhibits tumor growth, induces tumor regression in multiple HR+/HER2- xenograft models



BTX-9341 exhibits dose-dependent tumor growth inhibition in two HR+/HER2- xenograft models ZR-75-1 and T47D. Tumor growth inhibition at all doses of BTX-9341 is more potent than palbociclib and a lower dose of Abemaciclib, and in the T47D model, all doses are more potent than this higher dose of abemaciclib as well. Tumor regression is seen at all doses of BTX-9341 tested.

CONCLUSIONS

These preclinical data show that BTX-9341 promotes specific, CRBN and proteasome dependent degradation of CDK4 and CDK6 in multiple breast cancer cell lines. This degradation leads to a deeper and more sustained inhibition of cell cycle progression, phospho-Rb, E2F target gene expression and cell proliferation when compared to CDK4/6i. BTX-9341 exhibited more potent tumor growth inhibition in multiple HR+/HER2- xenograft models compared to CDK4/6i and induced tumor regression at some doses. BTX-9341 was efficacious in a palbociclib-resistant cell line indicating that a degrader approach may work well in patients who are resistant to CDK4/6 inhibitors. Considering these properties, we are in the process of filing an IND for BTX-9341.

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