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

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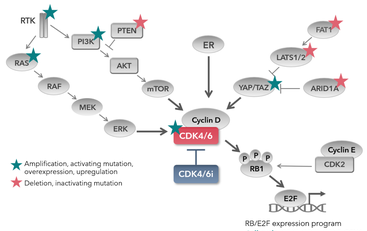
## BACKGROUND




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 HR+/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.
- 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

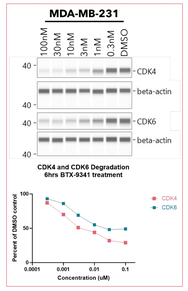
- BTX-9341 is a potent, CRBN dependent degrader of CDK4 and CDK6 in multiple breast cancer cell lines.
- Kinome profiling indicates BTX-9341 is more selective than the CDK4/6i palbociclib at 100 nM, and proteomics indicates minimal off-target degradation.
- BTX-9341 inhibits Rb phosphorylation and E2F target gene expression leading to and Inhibition of proliferation, with colony formation assay $IC_{50}$s in the low nanomolar range.
- BTX-9341 maintains Rb phosphorylation inhibition and proliferation inhibitor in a T47D palbociclib resistant cell line.
- BTX-9341 exhibits sustained inhibition of Rb phosphorylation and E2F target gene expression, while CDK4/6 inhibitors show recovery
- BTX-9341 exhibits synergy with the selective estrogen receptor degraders (SERDs) in a colony formation assay.
- BTX-9341 exhibits enhanced synergy with SERDs in a palbociclib resistant cell line as compared to CDK4/6 inhibitors in combination with SERDs in this 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 correlated with CDK4, CDK6 and pRb downregulation.
- BTX-9341 also inhibits tumor growth in several other HR+/HER2- xenograft models.

**REFERENCES**

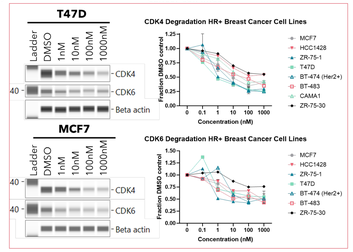
1. Scheidemann, E.R., et al. *Int J Mol Sci* **22**, 12292 (2021).
2. Álvarez-Fernández, M., et al. *Cancer Cell* **37**, 514-529 (2020).
3. Li, Z., et al. *Cancer Cell* **34**, 893-904 (2018).
4. Li, Q., et al. *Cancer Discovery* **12**, 356-371 (2022).
5. Razavi, P., et al. *Cancer Cell* **34**, 427-438 (2018).

## BTX-9341 degrades CDK4 and CDK6 with minimal off-target binding or degradation

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

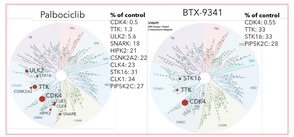



**Degradation of CDK4 and CDK6 at 6 hours of treatment HR+ breast cancer cell lines**




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-.

**Kinome selectivity profiling of BTX-9341**

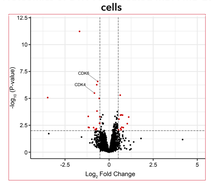



Kinome S(35) score: the fraction of kinases with less than 35% of control at 100nM among the 468 kinases tested with the KINOMEscan™ platform from Eurofins.

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

| Compound | CDK6 Kd |
|---|---|
| BTX-9341 | 31 nM |
| Palbociclib | 5.1 nM |

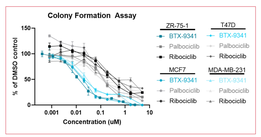
**Proteomics of BTX-9341 treated MDA-MB-231 cells**




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.

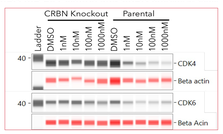
## BTX-9341 inhibits RB phosphorylation and cell proliferation

### BTX-9341 inhibits cell proliferation

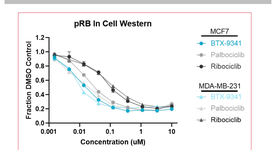



### BTX-9341 activity is dependent on Cereblon

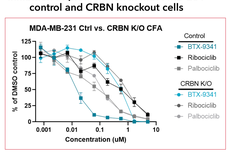
**Degradation of CDK4/CDK6 at 6 hours in MDA-MB-231 control and CRBN knockout cells**



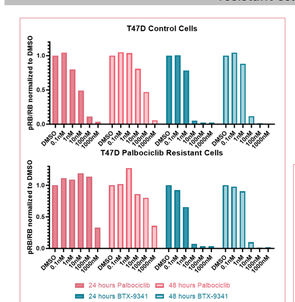

### BTX-9341 inhibits Rb phosphorylation



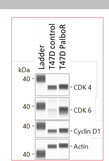

**Inhibition of proliferation in MDA-MB-231 control and CRBN knockout cells**

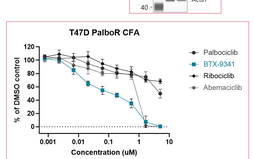



### BTX-9341 inhibits cell proliferation and Rb phosphorylation in a palbociclib resistant cell line



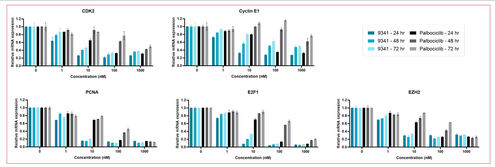

Inhibition of Rb phosphorylation over time measured by immunoblot after treatment of T47D control and palbociclib resistant cells with BTX-9341 or palbociclib.



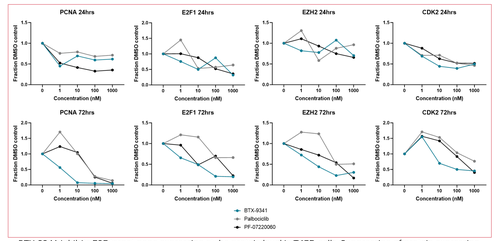




## BTX-9341 downregulates E2F target genes and pRb in a rapid and sustained manner

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

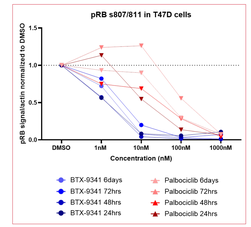


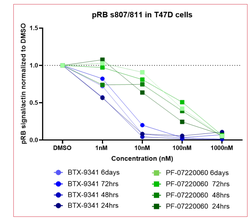

BTX-9341 inhibits E2F target gene mRNA expression in T47D cells. Suppression of gene expression is sustained through 72 hours, while recovery of gene expression is seen starting at 48 hours after palbociclib treatment




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

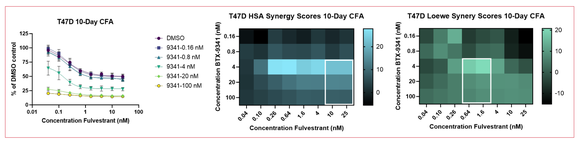
### Inhibition of pRb is more sustained with BTX-9341 treatment than CDK4/6i

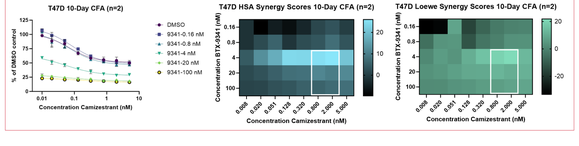



## BTX-9341 exhibits strong synergy with SERDs in HR+/HER2- T47D cells and T47D cells resistant to palbociclib

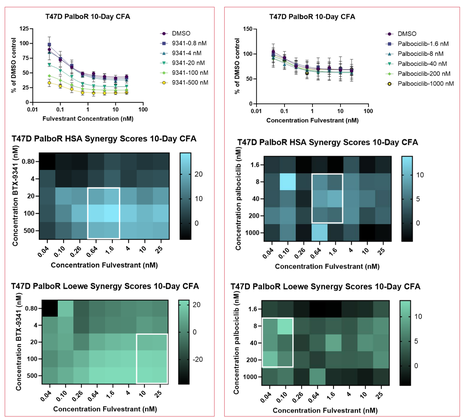
### BTX-9341 exhibits synergy with SERDs in T47D cells




| T47D cells | | Synergy score | MSA |
|---|---|---|---|
| 9341+ fulvestrant | HSA | 8.19 | 15.48 |
| | Loewe | 3.19 | 11.02 |
| 9341+ camizestrant | HSA | 5.94 | 14.14 |
| | Loewe | 2.46 | 12.59 |
| 9341+ palazestrant | HSA | 6.57 | 13.82 |
| | Loewe | 1.25 | 10.06 |
| 9341+ vepdegestrant | HSA | 5.96 | 12.51 |
| | Loewe | 1.57 | 8.52 |

### BTX-9341 exhibits better synergy with SERDs than CDK4/6i in T47D palbociclib resistant cells



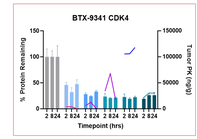


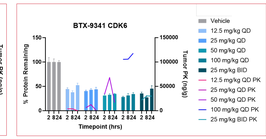


| T47D PalboR cells | | Synergy score | MSA |
|---|---|---|---|
| **9341+ fulvestrant** | **HSA** | **10.96** | **21.62** |
| | **Loewe** | **12.49** | **24.07** |
| Palbociclib+ fulvestrant | HSA | 3.26 | 6.46 |
| | Loewe | 3.40 | 8.11 |
| PF-07220060+ fulvestrant | HSA | 5.10 | 10.26 |
| | Loewe | 1.50 | 9.01 |
| **9341+ camizestrant** | **HSA** | **8.24** | **18.89** |
| | **Loewe** | **7.34** | **18.27** |
| Palbociclib+ camizestrant | HSA | 6.37 | 14.81 |
| | Loewe | 2.52 | 12.02 |
| PF-07220060+ camizestrant | HSA | 4.71 | 11.29 |
| | Loewe | 1.10 | 10.21 |
| **9341+ palazestrant** | **HSA** | **11.51** | **22.67** |
| | **Loewe** | **10.35** | **22.40** |
| Palbociclib+ palazestrant | HSA | 5.95 | 12.99 |
| | Loewe | -1.38 | 8.94 |
| PF-07220060+ palazestrant | HSA | 2.72 | 10.21 |
| | Loewe | 1.39 | 9.33 |
| **9341+ vepdegestrant** | **HSA** | **10.35** | **23.30** |
| | **Loewe** | **9.28** | **21.50** |
| Palbociclib+ vepdegestrant | HSA | 3.86 | 9.63 |
| | Loewe | 3.21 | 9.11 |
| PF-07220060+ vepdegestrant | HSA | 3.90 | 10.27 |
| | Loewe | 2.61 | 9.75 |

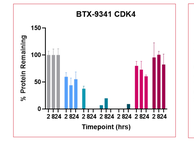
## BTX-9341 induces tumor regression in breast cancer xenograft models

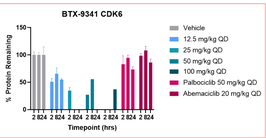
### BTX-9341 degrades CDK4, and CDK6, and inhibits pRb in MCF7subcutaneous tumors



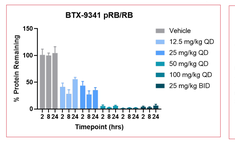



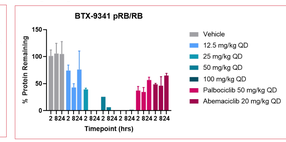

BTX-9341 exhibits dose-dependent target degradation of CDK4 and CDK6 that is rapid and sustained.



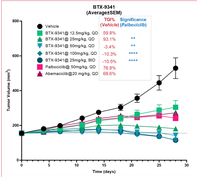

BTX-9341 exhibits dose-dependent target degradation of CDK4 and CDK6.



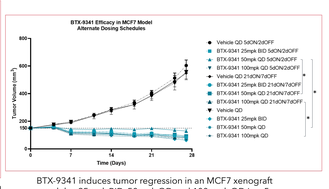




BTX-9341 exhibits a dose-dependent decrease in phosphorylated Rb relative to total Rb that is rapid and sustained. In an MCF7 xenograft efficacy model decreases in pRb are more significant than CDK4/6i at dose levels higher than 25mpk.

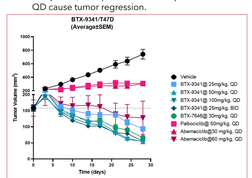
### BTX-9341 induces tumor regression in multiple HR+/HER2- breast caner xenograft models

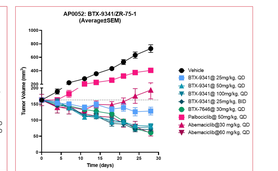



BTX-9341 exhibits dose-dependent tumor growth inhibition in an MCF7 xenograft model. BTX-9341 dosed at 25mpk BID, 50 mpk QD and 100 mpk QD cause tumor regression.




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.




BTX-9341 exhibits dose-dependent tumor growth inhibition in two HR+/HER2- xenograft models ZR-75-1 and T47D. Tumor regression is seen at all doses of BTX-9341 tested.

## CONCLUSIONS

These data show that BTX-9341 promotes specific, CRBN-dependent degradation of CDK4 and CDK6 in multiple breast cancer cell lines. This degradation leads to a deeper, more sustained inhibition of phospho-Rb, E2F target gene expression and cell proliferation when compared to CDK4/6i. BTX-9341 displayed synergy with SERDs that was maintained in palbociclib resistant cells, indicating a degrader approach in combination with a SERD may work well in patients resistant to CDK4/6 inhibitors. BTX-9341 exhibited potent tumor growth inhibition in multiple HR+/HER2- breast cancer xenograft models. Considering these properties, we have initiated a phase 1 clinical trial with BTX-9341 in HR+/HER2- breast cancer patients who have progressed after CDK4/6i therapy.

**Study Status**

- **BTX-9341-101 is currently active and recruiting participants [See Poster Presentation ID: P4-08-17]**
- **For more information on the study and sites, please visit www.clinicaltrials.gov (NCT06515470)**

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