# The Stapled Peptide ALRN-6924, a Dual Inhibitor of MDMX and MDM2, and the CDK4/6 Inhibitors Palbociclib, Ribociclib, or Abemaciclib Synergistically Enhance Each Other's *in vitro* and *in vivo* Anticancer Activity


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## Background

ALRN-6924 is a clinical-stage cell-permeating α-helical stapled peptide that disrupts the interaction of the p53 tumor suppressor protein and its inhibitors, MDMX and MDM2. Reactivation of p53 with ALRN-6924 in TP53-wild-type tumors triggers cell cycle arrest and apoptosis resulting in antitumor efficacy. CDK4/6 inhibitors (CDK4/6i's ) induce senescence and cell growth arrest via the interrelated Rb pathway, and co-amplification of MDM2 and CDK4 (both on chromosome 12q13) is a known oncogenic driver, suggesting that combinations of ALRN-6924 and CDK4/6i's may be synergistic. This study evaluates the antitumor efficacy and pharmacodynamics (PD) of ALRN-6924 combined with palbociclib, ribocivlib, or abemaciclib.

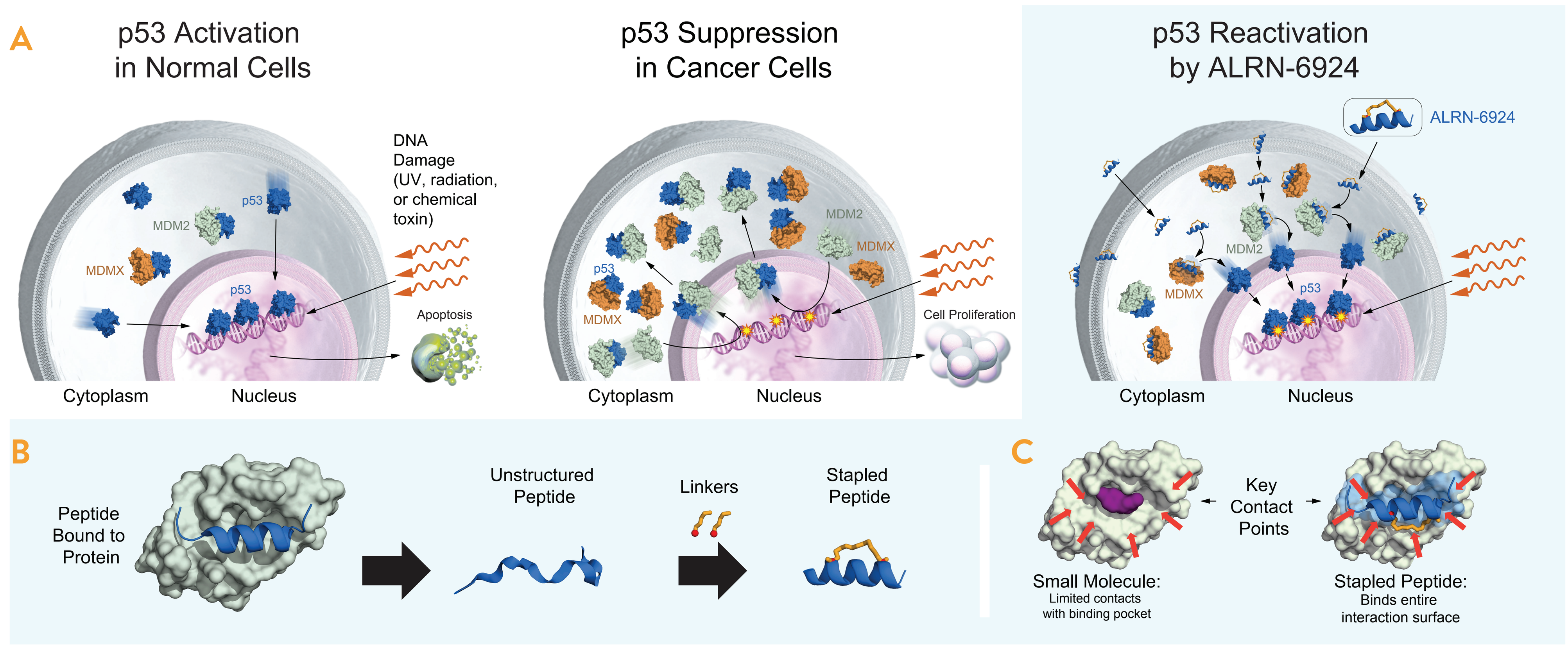
## Material and Methods

ALRN-6924 was tested in combination with palbociclib or abemaciclib in MCF-7 breast cancer cell lines and MDM2- and CDK4-co-amplified SJSA-1 sarcoma cell lines using WST-1 cell viability assays. Synergy was quantified by the Chou-Talalay combination index method. Single agents and combinations were evaluated in cell culture using assays for apoptosis (Caspase 3/7 cleavage), proliferation (BrdU), senescence (ß-Galactosidase), colony growth (Giemsa), and Western blot analysis of p53, p21, Rb, phospho-Rb, FOXM1, and phospho-FOXM1; and E2F1 mRNA. *In vivo* combinations with palbociclib, ribociclib, or abemaciclib were tested in athymic nude mouse MCF-7 and SJSA-1 xenograft models.

## Results

ALRN-6924 combinations with palbociclib or abemaciclib display synergistic *in vitro* antiproliferative activity in MCF-7 and SJSA-1 cells. ALRN-6924 induces senescence in vitro as a monotherapy and in combination with CDK4/6i's. Western blot assays show that ALRN-6924/palbociclib combinations trigger sustained on-mechanism biomarker activation, vs. transient activation with single agents. Phospho-Rb and phospho-FOXM1 down-regulation, p53 and p21 up-regulation, and repression of E2F1 mRNA are sustained after wash-out in combination, but not in single agent-treated cells. MCF-7 and SJSA-1 tumor growth inhibition was improved in mice treated with ALRN-6924 combinations with either palbociclib, ribociclib, or abemaciclib vs. single agent. EdU assays show that ALRN-6924/palbociclib combinations inhibit SJSA-1 tumor cell proliferation *in vivo*. Body weights and mortality data show the combination of ALRN-6924 with palbociclib or ribociclib 75 mg/kg/day was well tolerated; the combination with abemaciclib 100 mg/kg/day was tolerated with interruption and dose-reduction. No pharmacokinetic (PK) drug-drug interactions were noted in nude mice due to different modes of metabolism for ALRN-6924 (proteolysis) and palbociclib (CYP3A).

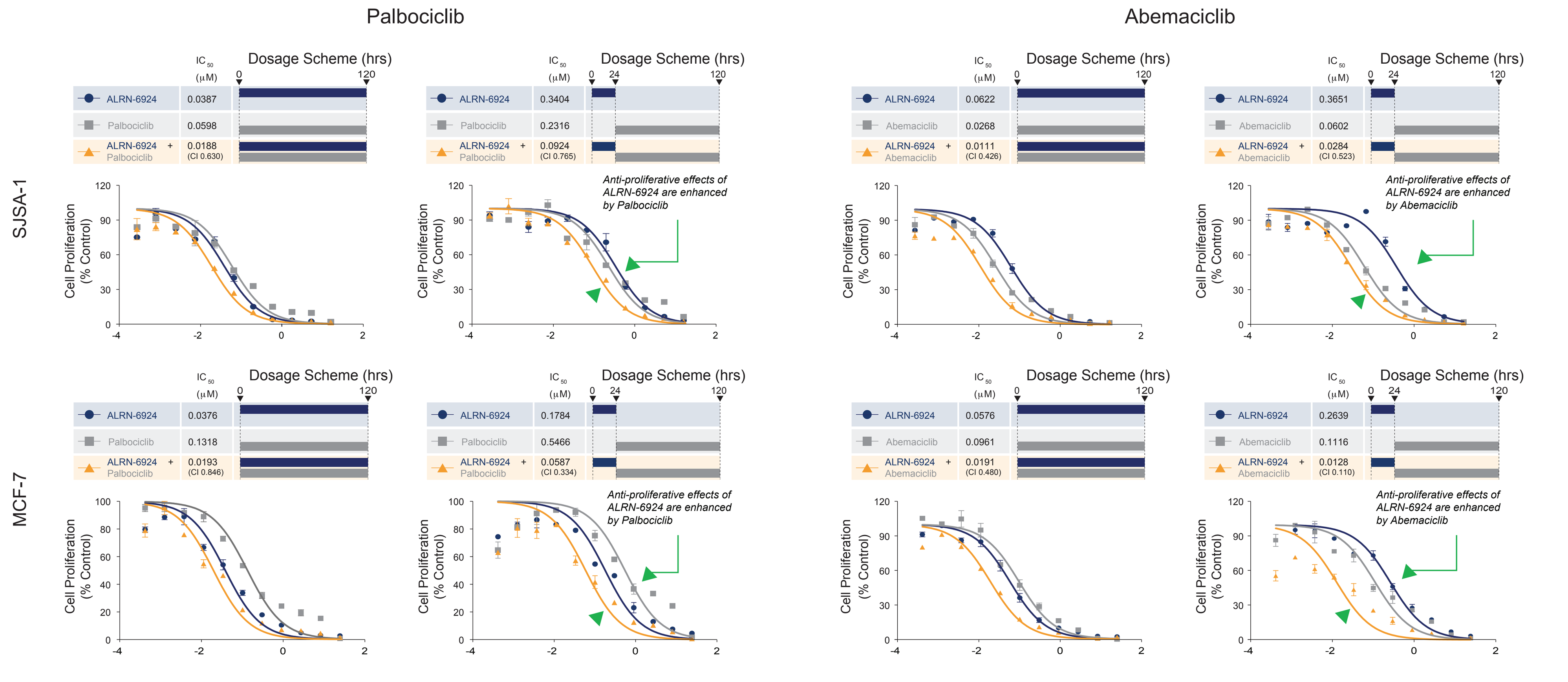
### Figure 1: The Cell-Permeating Stabilized α-helical Peptide, ALRN-6924, is a First-in-Class Dual Inhibitor of MDMX and MDM2



A
p53 Activation in Normal Cells
p53 Suppression in Cancer Cells
p53 Reactivation by ALRN-6924
ALRN-6924
DNA Damage (UV, radiation, or chemical toxin)
MDM2
MDMX
p53
Apoptosis
Cell Proliferation
Cytoplasm
Nucleus
B
Peptide Bound to Protein
Unstructured Peptide
Linkers
Stapled Peptide
C
Key Contact Points
Small Molecule: Limited contacts with binding pocket
Stapled Peptide: Binds entire interaction surface


A) The tumor suppressor p53 is one of the most pursued targets in oncology, playing a central role inducing cell cycle arrest, apoptosis, senescence, autophagy, cellular metabolism and immune surveillance in response to cellular stresses such as DNA damage and oncogenic signals[1]. B) ALRN-6924, a cell-permeating, stapled α-helical peptide that has demonstrated anticancer activity as monotherapy in clinical trials[2,3], mimics the p53 tumor suppressor protein to disrupt its interactions with both its endogenous inhibitors, MDMX and MDM2[4,5]. Stapled peptides mimic natural peptide sequences at the interface of protein-protein interactions, displaying a larger surface area of interaction with its target, and providing superior binding properties which reduce off-target effects and the risk of acquiring mutations associated with resistance. C) Furthermore, like natural protein sequences, a peptide can engage with ≥2 targets, e.g. MDMX + MDM2.

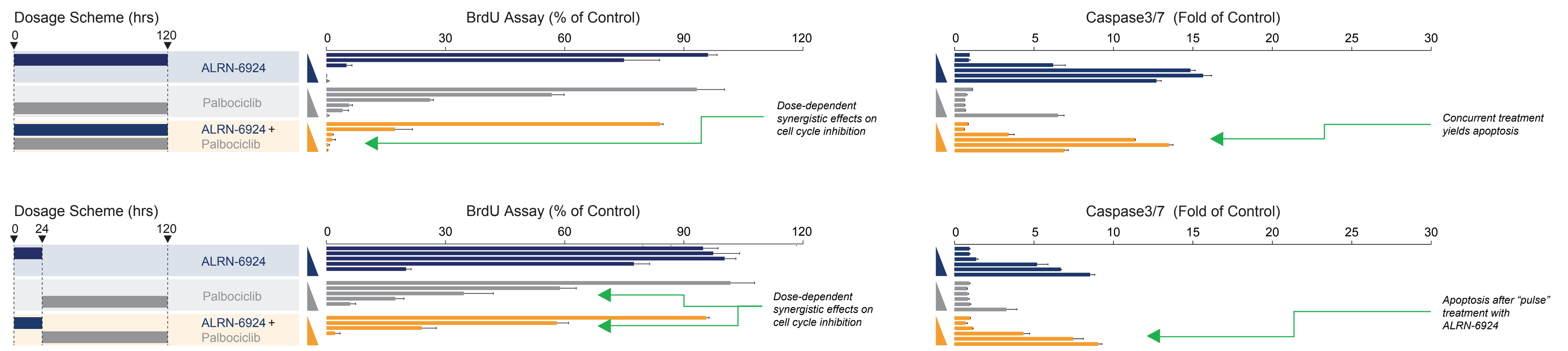
### Figure 2: ALRN-6924 in Combination with CDK4/6i's Displays Synergistic Anti-proliferative Activity *in vitro*



Palbociclib
Abemaciclib
SJSA-1
MCF-7
Dosage Scheme (hrs)
IC50 (µM)
Cell Proliferation (% Control)
Anti-proliferative effects of ALRN-6924 are enhanced by Palbociclib
Anti-proliferative effects of ALRN-6924 are enhanced by Abemaciclib


Synergy is observed on concomitant dosing of ALRN-6924 and palbociclib or abemaciclib. Sustained antiproliferative effects of ALRN-6924 "pulse" are enhanced by palbociclib or abemaciclib A pulse of palbociclib or abemaciclib in MCF-7 shows added benefit over ALRN-6924 alone.

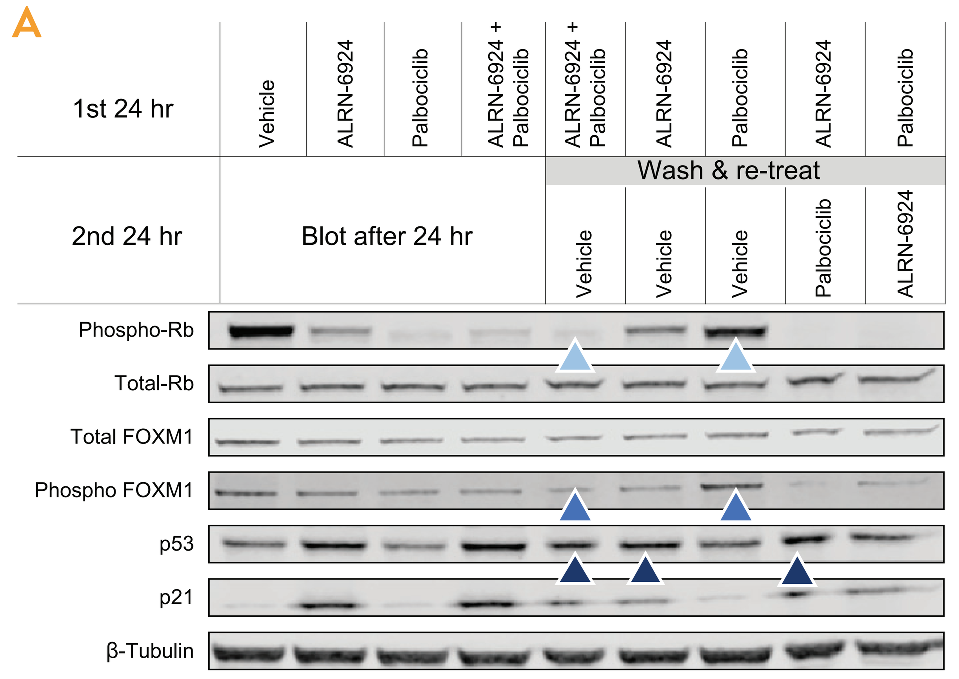
### Figure 3: ALRN-6924 in Combination with Palbociclib Displays Synergistic Cell Cycle Inhibition and Induces Apoptosis



Dosage Scheme (hrs)
BrdU Assay (% of Control)
Caspase3/7 (Fold of Control)
ALRN-6924
Palbociclib
ALRN-6924 + Palbociclib
Dose-dependent synergistic effects on cell cycle inhibition
Concurrent treatment yields apoptosis
Apoptosis after "pulse" treatment with ALRN-6924


BrdU incorporation and caspase 3/7 apoptosis assays in SJSA-1 osteosarcoma cell lines when treated with ALRN-6924 and palbociclib over a 3-fold dilution range of either agent (or both) from 0.2 to 16.2 µM.

### Figure 4: The Combination of ALRN-6924 and Palbociclib Triggers Sustained Activation of the RB Tumor Suppressor Pathway

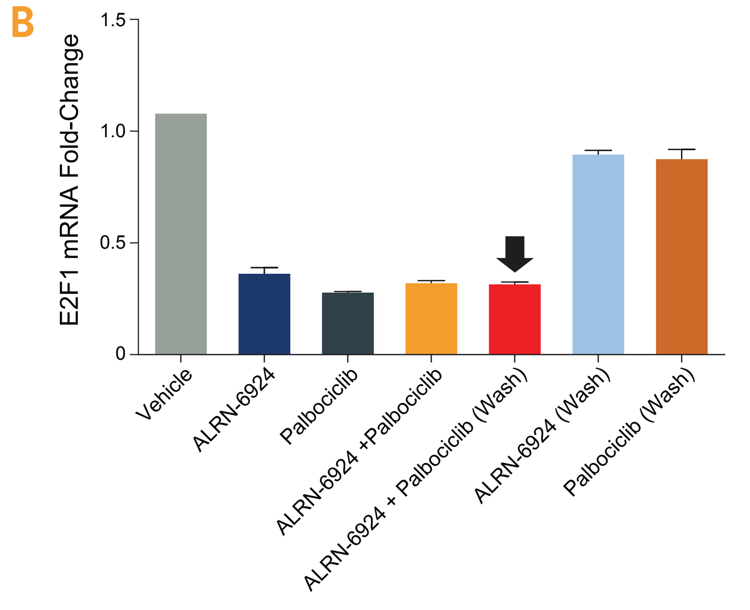


A
1st 24 hr
2nd 24 hr
Blot after 24 hr
Wash & re-treat
Vehicle
ALRN-6924
Palbociclib
ALRN-6924 + Palbociclib
Phospho-Rb
Total-Rb
Total FOXM1
Phospho FOXM1
p53
p21
β-Tubulin


Combination treatment in SJSA-1 cells sustains phospho-Rb down-regulation after wash-out.

Combination treatment sustains phospho-FOXM1 down-regulation after wash-out

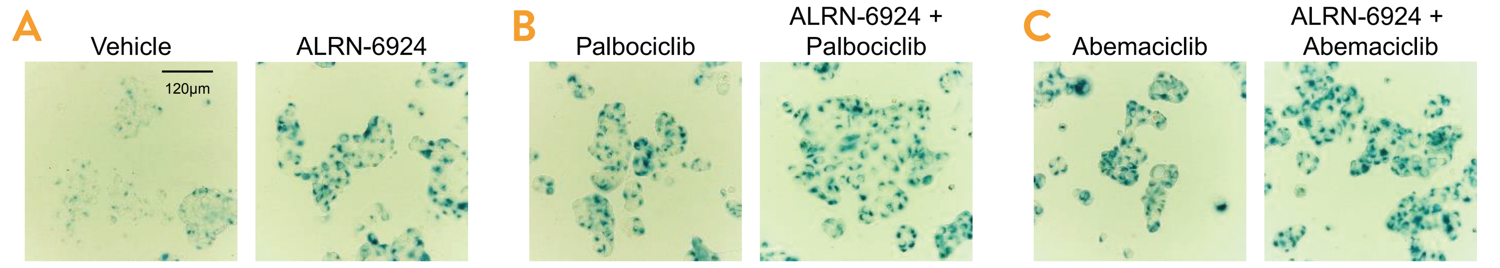
ALRN-6924 sustains p53 and p21 up-regulation after wash-out



B
E2F1 mRNA Fold-Change
Vehicle
ALRN-6924
Palbociclib
ALRN-6924 +Palbociclib
ALRN-6924 + Palbociclib (Wash)
ALRN-6924 (Wash)
Palbociclib (Wash)


Combination treatment in SJSA-1 cells yields sustained repression of E2F1 following wash-out

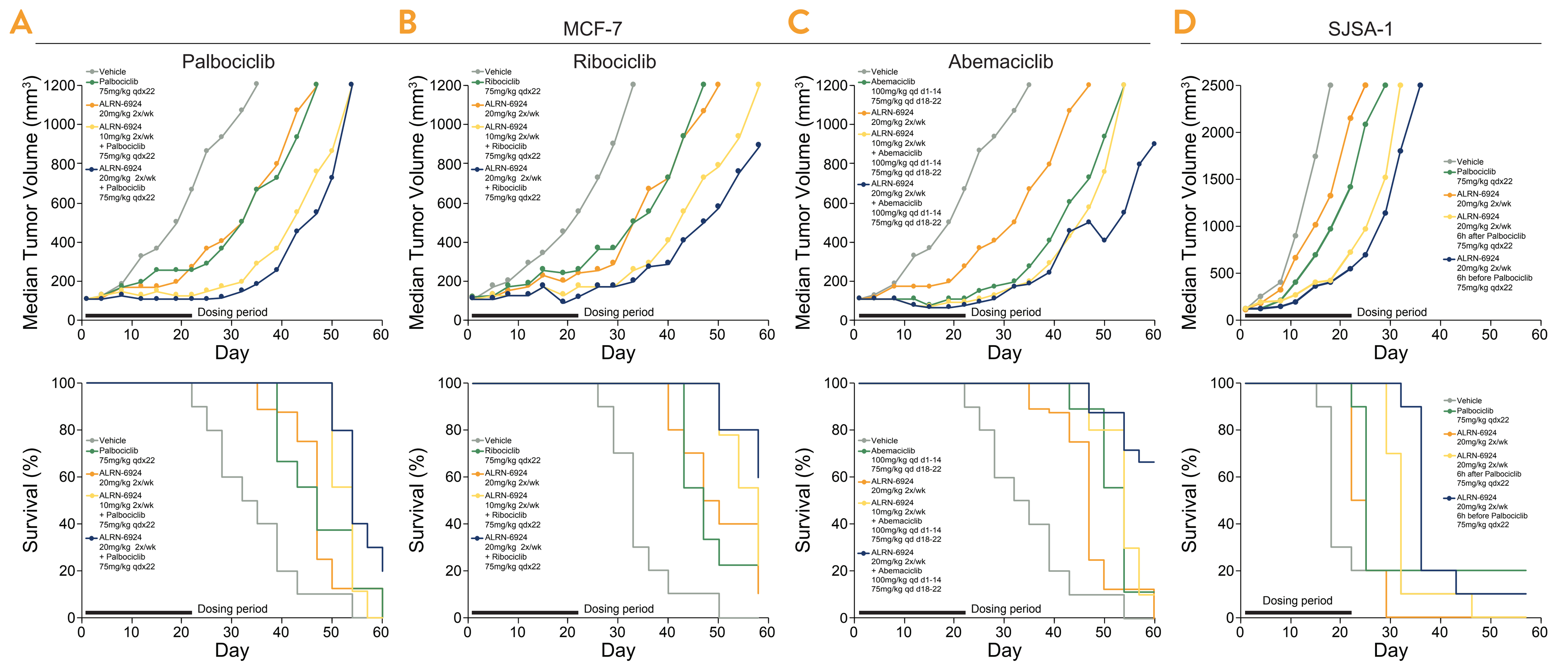
### Figure 5: ALRN-6924 Induces Senescence *in vitro* as a Monotherapy and in Combination with CDK4/6i's



A
Vehicle
ALRN-6924
120µm
B
Palbociclib
ALRN-6924 + Palbociclib
C
Abemaciclib
ALRN-6924 + Abemaciclib


MCF-7 breast cancer cells treated with A) ALRN-6924 (0.3 µM) alone or in combination with B) palbociclib (0.3 µM) or C) abemaciclib (0.3 µM), for seven (7) days, then washed and visualized with ß-galactosidase substrate X-Gal.

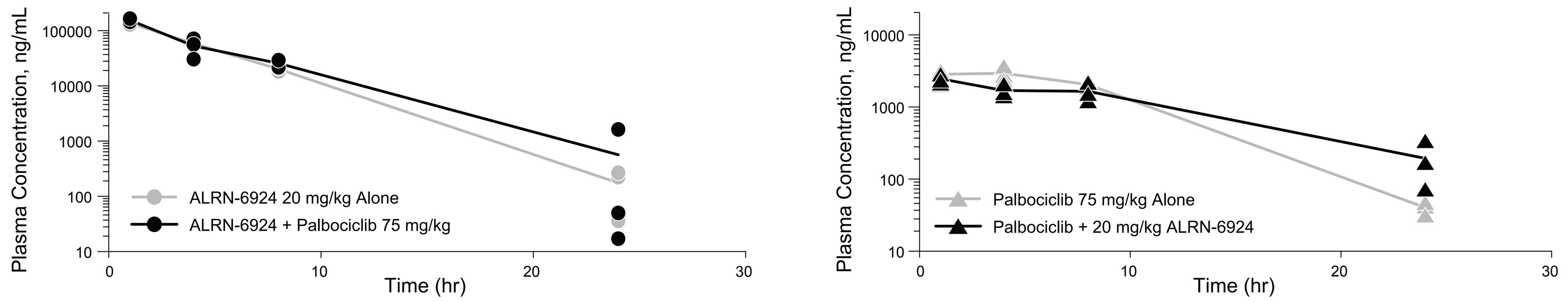
### Figure 6: ALRN-6924 Combinations with CDK4/6i's Yield Enhanced Anti-tumor Activity *in vivo*



A
B
MCF-7
C
D
SJSA-1
Palbociclib
Ribociclib
Abemaciclib
Median Tumor Volume (mm³)
Survival (%)
Day
Dosing period


Athymic nude mice with established MCF-7 breast cancer tumors were treated with ALRN-6924 alone (intravenously twice weekly for 3 weeks) or in combination with A) palbociclib or B) ribociclib 75 mg/kg orally daily for 22 days. C) abemaciclib was dosed 100 mg/kg orally daily 14 days, then after a brief treatment holiday due to weight loss, dosing resumed on day 18 at 75 mg/kg to day 22. D) ALRN-6924/palbociclib single-agent and combination treatment as in A. in the SJSA-1 osteosarcoma model, with ALRN-6924 dosed either 6h before or after palbociclib. Studies were approved by the Institutional Animal Care and Use committee at Charles River Laboratories, Morrisville, N.C.

### Figure 7: No Pharmacokinetic Drug-Drug Interactions Are Observed in nu/nu Mice



Plasma Concentration, ng/mL
Time (hr)
ALRN-6924 20 mg/kg Alone
ALRN-6924 + Palbociclib 75 mg/kg
Palbociclib 75 mg/kg Alone
Palbociclib + 20 mg/kg ALRN-6924


No interaction is observed due to different disposition of ALRN-6924 (biliary) and Palbociclib (CYP3A).

## Conclusions

This study demonstrates that ALRN-6924 and CDK4/6i combinations show synergistic activity. PD biomarkers indicate on-mechanism *in vitro* activity that is sustained after wash-out. *In vivo* efficacy, biomarker, PK, and tolerability results, plus clinical evidence that the most frequent and concerning safety issues for CDK4/6i's (neutropenia, leukopenia, infections) do not overlap with ALRN-6924's reported safety profile[3] support the development of combination regimens for breast cancer and other malignancies. A Phase 1B trial to evaluate the combination of ALRN-6924 and palbociclib in MDM2-amplified and MDM2/CDK4-coamplified cancers will start enrolling patients in the first quarter of 2019 (ClinicalTrials.gov identifier NCT-02264613)

## References


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