# Potent anticancer activity against both BRAF mutant and BRAF wild-type melanoma cell lines using a novel CRM1 nuclear export inhibitor.

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# INTRODUCTION

First depicted three centuries ago, the nucleus becomes the first organelle discovered. This space within a cell is confined by a nuclear lamina and an inner and outer nuclear membrane, which separates the nuclear content from the surrounding cytoplasm serving as a barrier for macromolecules. The confinement of biomolecules within compartments is crucial for the formation and function of the cell. However, molecules do travel among cellular compartments. Regulatory proteins are constantly traveling across compartments to exert their function. An example would be tumor supressor p53, normally exported to the cytoplasm for degradation, under cellular stress p53 becomes trapped in the nucleus. Other regulatory factors exported from the nucleus include APC, p21, p27, Hdm2. Thus, the inhibition of nuclear export would seem like a possible target for cancer therapy. The idea has been around for more than 20 years, and has demostrated potent antitumoral activity across a broad range of tumors, including Malignant Melanoma. However, due to its toxicity, it failed at the phase I trial. We present novel selective inhibitors of nuclear export (SINE) and their effects on both BRAF mutant and wild-type melanoma cell lines as single therapy or in combination with BRAF inhibition.

AACR 2012 Poster Session. Abstract #1914

# MATERIALS AND METHODS

## Cell lines and reagents

Malignant melanoma cell lines were kindly donated by Dr. Hensin Tsao and Dr. Jennifer Wargo. All cell lines were maintained in DMEM high glucose with L-glutamine and supplemented with 10% Fetal Bovine Serum. Refer to table 1 for cell lines and mutations.

## Compounds

PLX4032 and PLX4720, selective, small molecule BRAF inhibitor were purchased from Selleck Chemicals. KPT251 and KPT276, selective inhibitors of nuclear export (SINEs), were supplied by Karyopharm Therapeutics.

## Cell Proliferation Assay

Cellular proliferation was evaluated by MTT assay and read at 72 hours. The IC50 and Combination Index Value (CIV) by Chou-Talaly were determined from the linear regression using Calcusyn Software (Biosoft) v1.1.

## Apoptosis Assay

Apoptosis was determined by Western Blot for PARP Cleavage and Cleaved Caspase-3 and quantified by Caspase-3/7 Elisa. Combination Index Value (CIV) by Chou-Talaly, were determined from the regression using by Calcusyn Software (Biosoft) v1.1.

## Cell Cycle Analysis

Cell cycle analysis was done by flow cytometry using propidium iodide (PI) and data was analyzed using WinMDI v2.9.

**Melanoma Cell Lines & Mutational Status**

| | *NRAS* | *PTEN* | *BRAF* |
|---|---|---|---|
| Effron | Wt | Wt | V600 |
| TC2861 | Wt | Wt | V600 |
| Mel-624 | Wt | Wt | V600 |
| A375 | Wt | Wt | V600 |
| Mel-28 | Wt | T167A | V600 |
| UAC903 | Wt | Tyr76Stp | V600 |
| PTEN | Wt | Deficient | V600 |
| SK-MEL-5 | Wt | Wt | V600 |
| G361 | Wt | Wt | V600 |
| TC2365 | Wt | ? | V600 |
| HS940T | Q61R | Wt | Wt |
| Mel-Juso | Qg1L | Wt | Wt |
| MeWo | WT | Wt | Wt |

**Table 1. Melanoma cell lines and mutational status.**

## Xenograft Model

Cell lines A375, Mel-Juso and MeWo were used for the xenograft model. Briefly, 5x10^6 cells in 200 ul of PBS were injected sc into the animal's flank and were allowed to grow before starting treatment until tumor volume reached 250-400 mm^3. Each treatment group was composed of 10 mice. A375 mice were divided in 4 groups of 10 mice. Each group was treated with Vehicle (Oral, ip or both), PLX4720 ip daily at 50 mg/kg , KPT276 PO every other day at 75 mg/kg or in by combination for 14 days. Mel-Juso and MeWo were treated for 14 days by vehicle PO or KPT276 every other day at 75 mg/kg for 21 days.

## Immunohistochemistry (IHC)

Tumors were collected from the mice 24 hours after treatment and cell dead and proliferation were assessed by IHC for cell Caspase-3 and Ki67, respectively.

## Immunofluorescence(IF)

IF was done for p53 in treated cell lines and DAPI was used as counter staining.

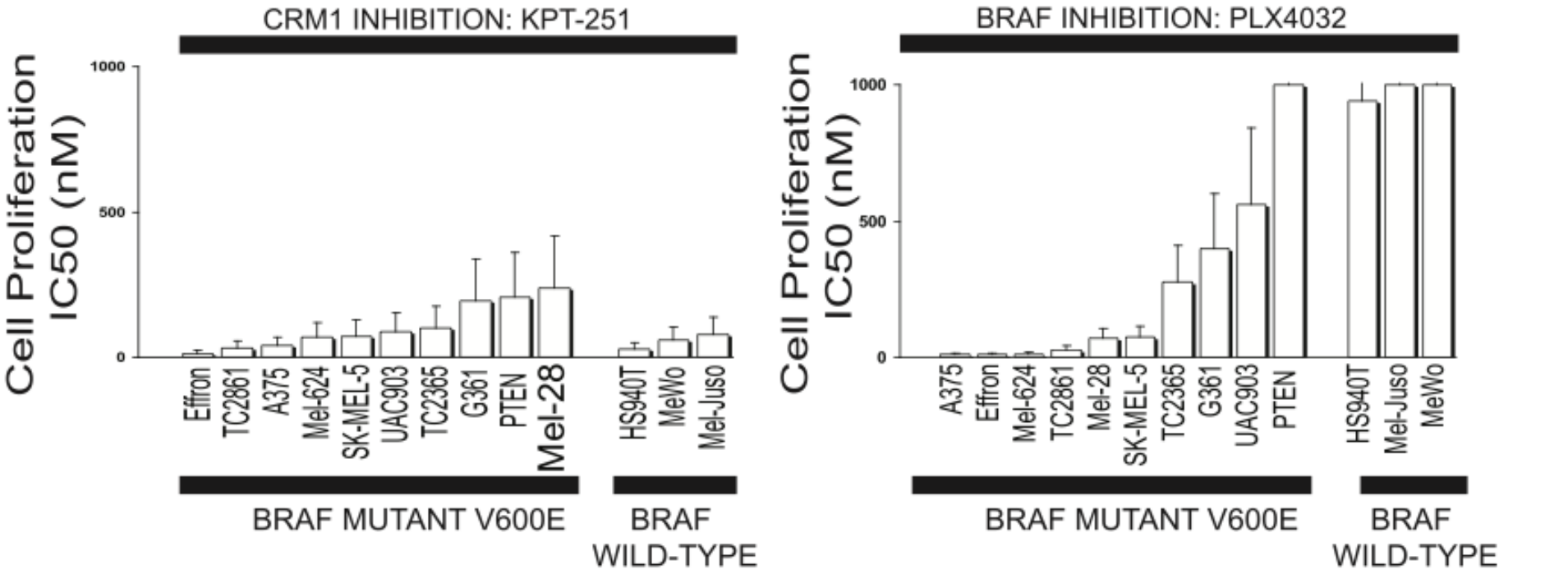



**Figure 1. MTT cell proliferation assay of melanoma cell lines after CRM1 or BRAF inhibition.**
*Inhibition of proliferation of tumor cell lines by KPT-251 is independent of BRAF, NRAS or PTEN mutational status in this group of melanoma cell lines and its effect across all tested cell lines is comparable to PLX4032 inhibitory effect on BRAF V600E mutant lines. Measurements were done after 72 hours and the corresponding IC50 for each cell line was determined by linear regression using Calcusyn v1.1.*

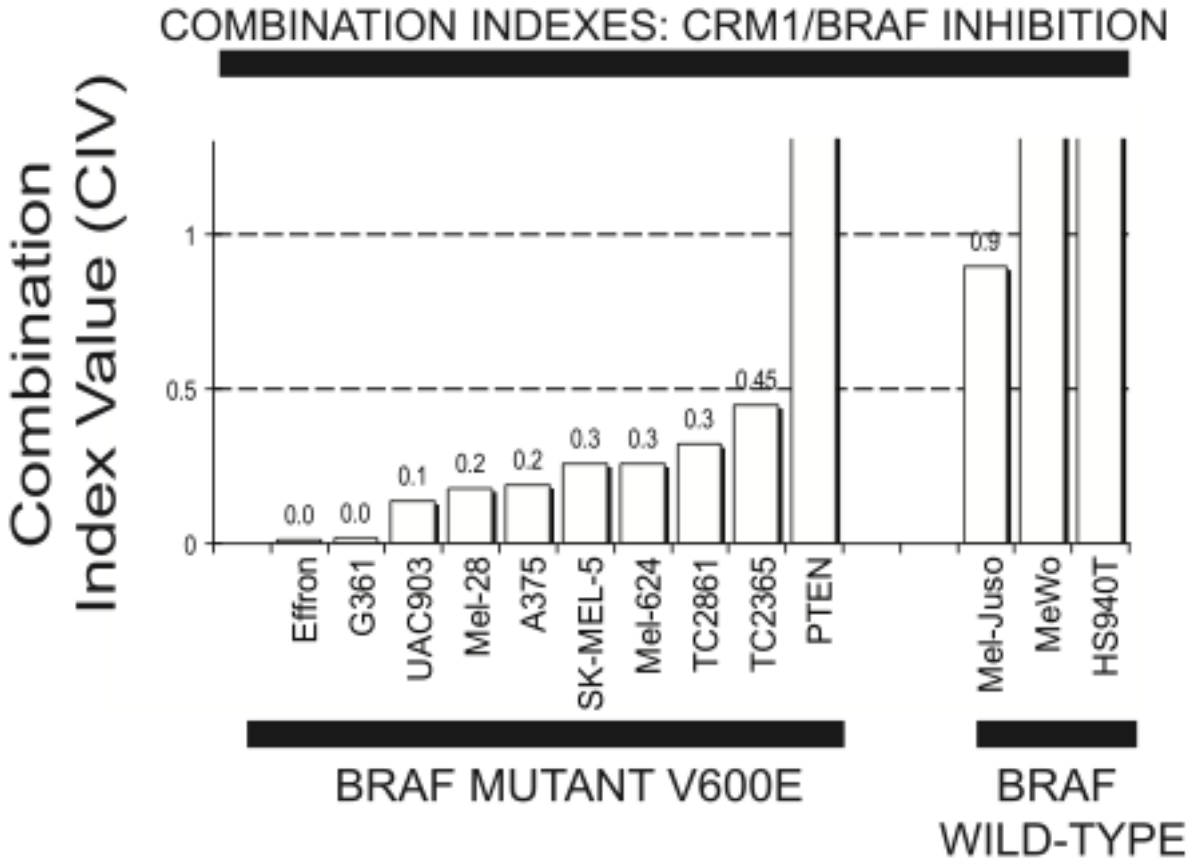



**Figure 2. Combination Index Value (CIV) across melanoma cells lines after 72 hours of CRM1 and BRAF inhibition with KPT-251 and PLX4032, respectively, at 1:1 ratio.**

*A CIV lower than 1 denotes synergy, with strong synergy being observed in combination with a CIV < 0.3. A CIV > 1 denotes an antagonistic effect.*

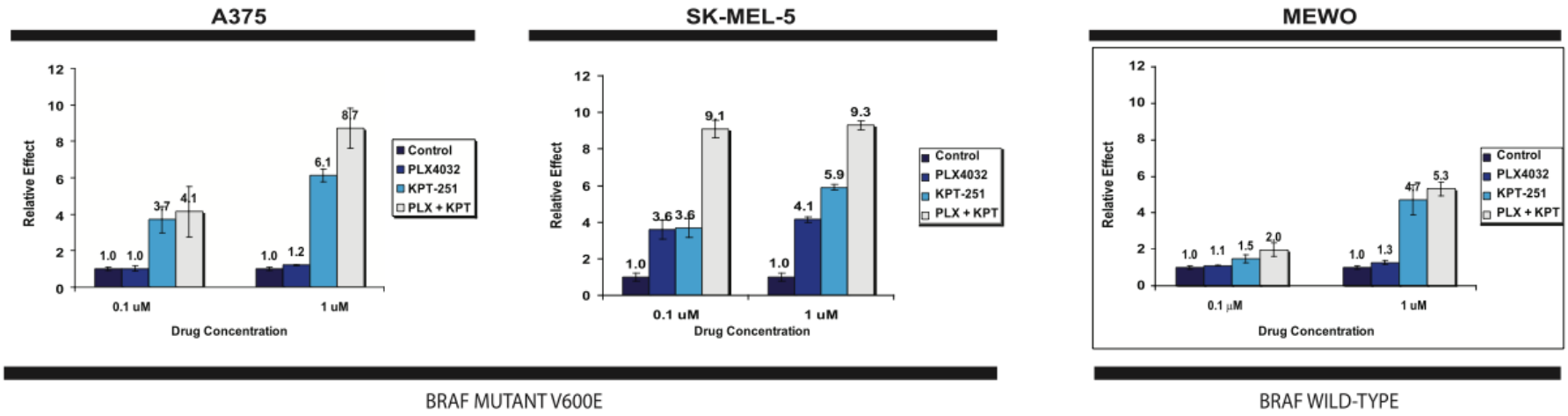



**Figure 3. Apoptotic effect of CRM1 inhibition, BRAF inhibition and combination on apoptosis in human melanoma cell lines.**

*ELISA for Caspase-3/7 in melanoma cell lines showing an increase in the apoptotic marker at 0.1 uM and 1 uM for both BRAF and CRM1 inhibitors, PLX4032 and KPT-251, respectively and their combination at 24 hours. CIV < 0.3 (Cell lines tested: A375, SK-Mel-5 and Mel-Juso)*

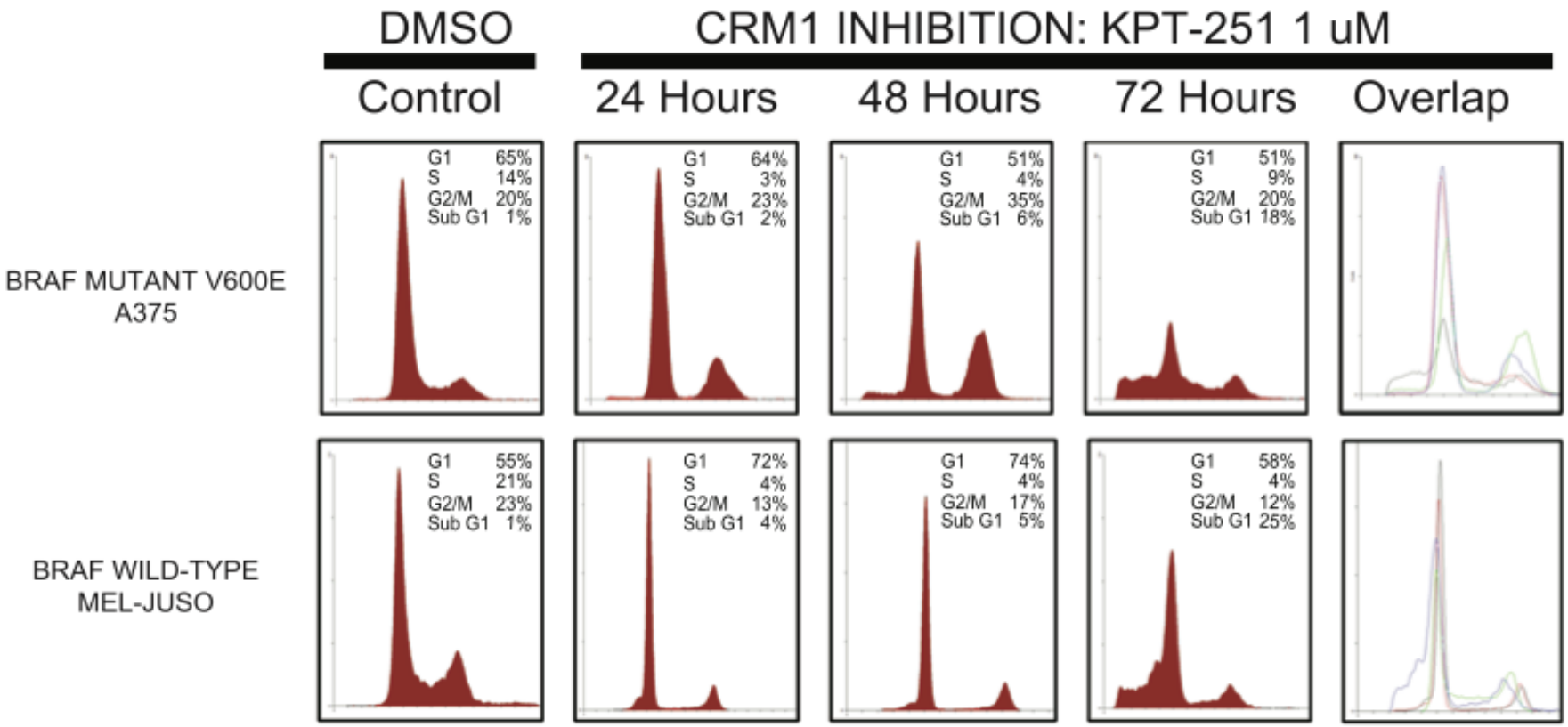



**Figure 4 . Cell cycle effect of CRM1 inhibition on melanoma cell lines.**

*Cell cycle analysis using PI was performed at 24, 48 and 72 hours after CRM1 inhibition using KPT-251 at 1 uM on cell lines A375 and Mel-Juso, BRAF mutant and wild-type, respectively. In both cell lines, CRM1 inhibition leads to a significant decrease in S phase during the first 24 hours. Marked G1 arrest in Mel-Juso and a G1 and G2 arrest in A375 is also observed as time progresses. After 48 hours, marked apoptosis is observed in both cell lines, with a sub-G1 ranging from 20-25%.*

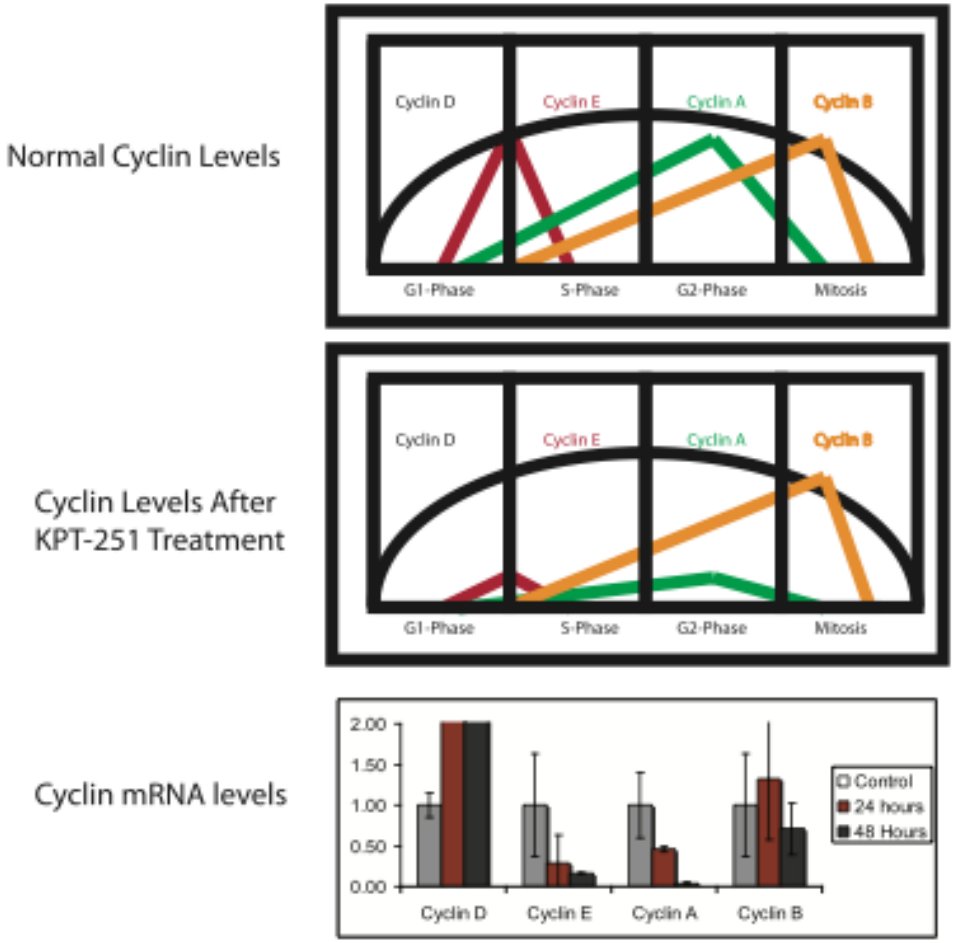



**Figure 5. CRM1 inhibition effect on mRNA levels of melanoma cell lines after 24 and 48 hours of treatment.**

*Using qRT-PCR, cyclin levels were determined at 24 and 48 hours. Cyclin E and A, which are required for progression into and out of S phase were decreased, correlating with the observed findings in the cell cycle.*

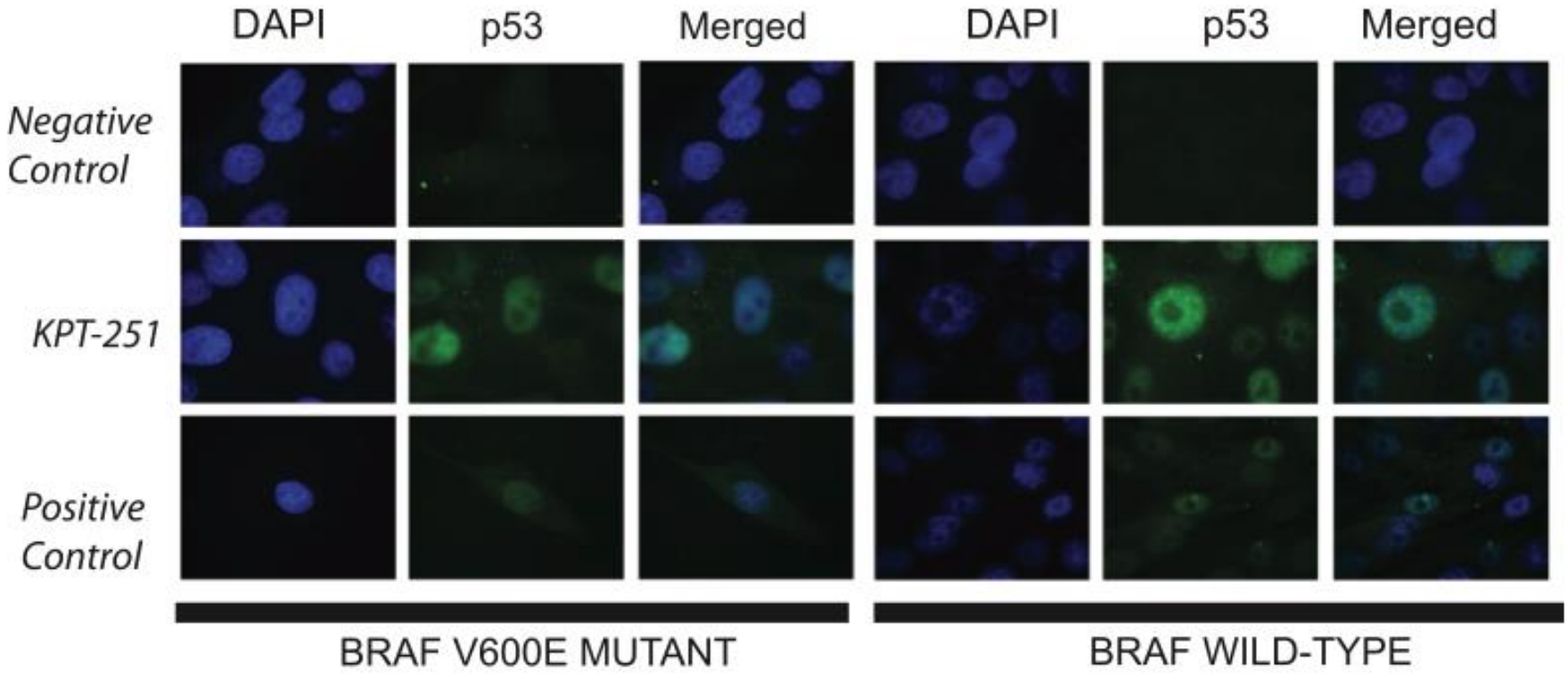



**Figure 6. Immunofluorescence showing nuclear localization of p53 after 24 hours of CRM1 inhibition in both BRAF mutant and wild-type melanoma cell lines.** *Nuclear localization of p53 is required for the protein to exert it's functions. Nuclear localization of p53 prevents it's cytoplasmic degradation. Melanoma cell lines were treated with KPT-251 at 1 uM for 24 hours.*

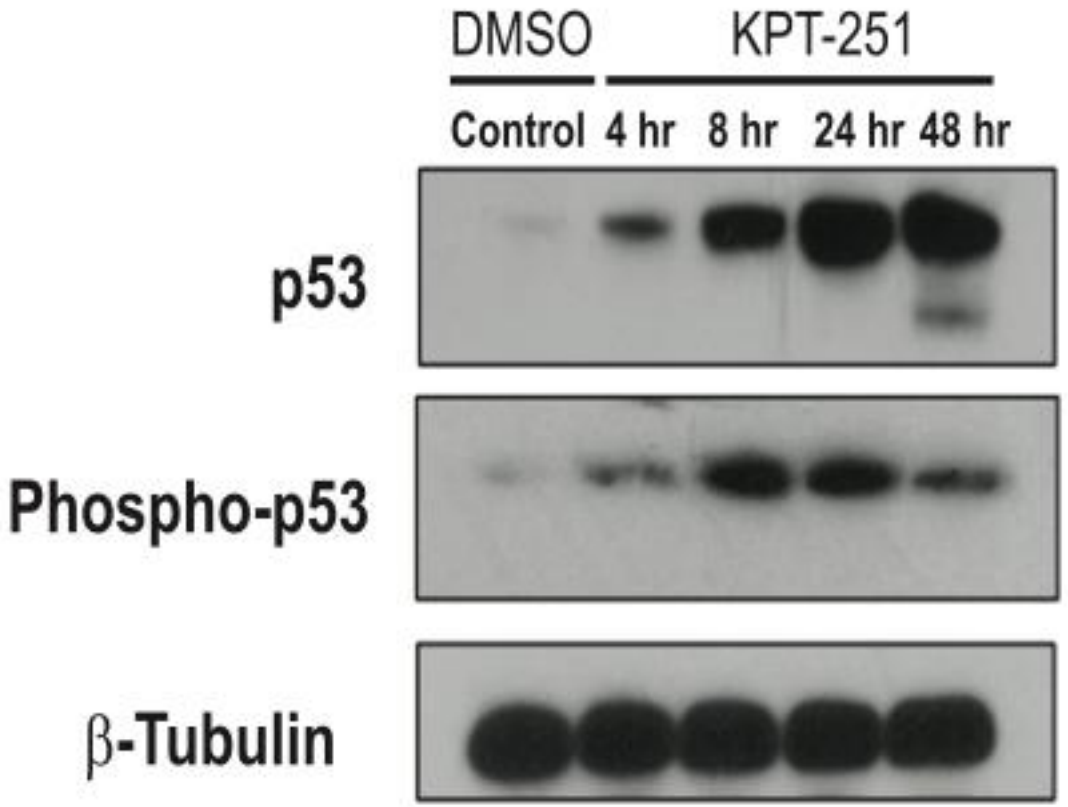



**Figure 7. Western blot showing increase in p53 and phospho-p53 after CRM1 inhibition in a time-dependent manner.**

*Using KPT-251 1 uM; Effect seen on both BRAF mutant and wild-type melanoma cell lines.*

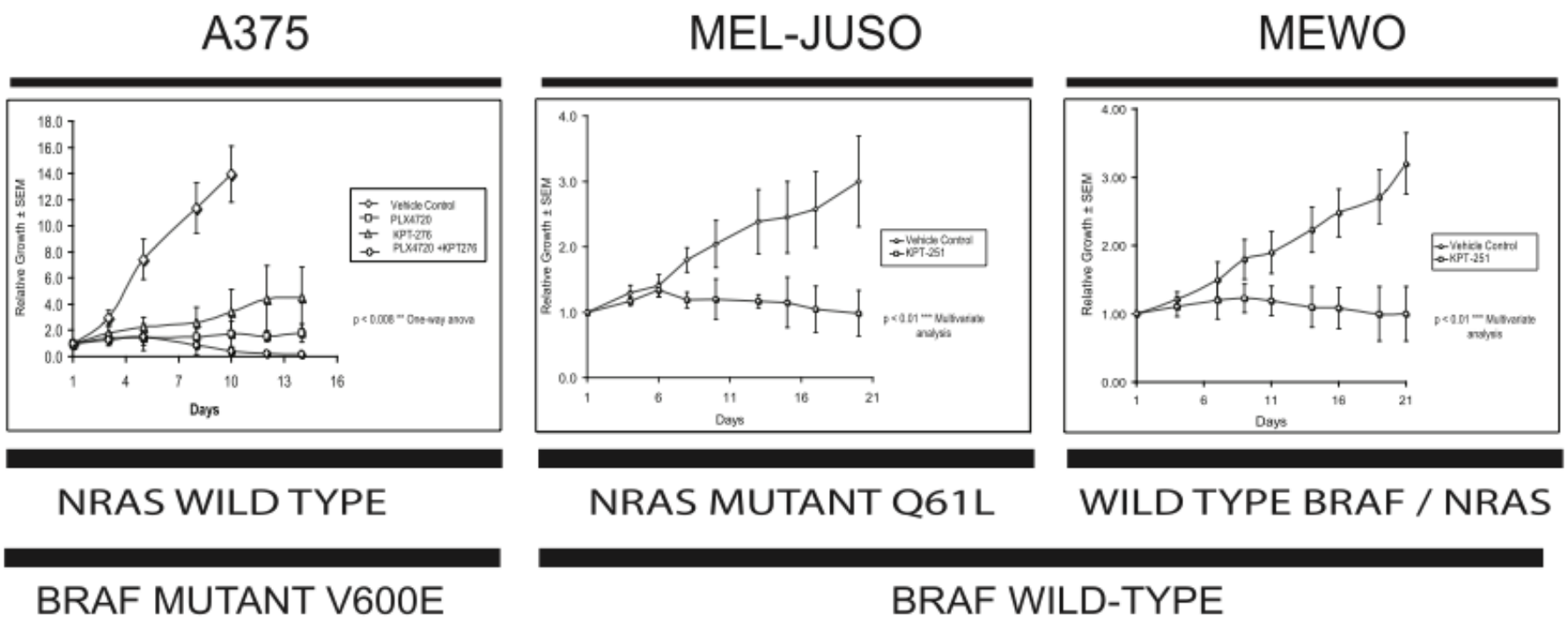
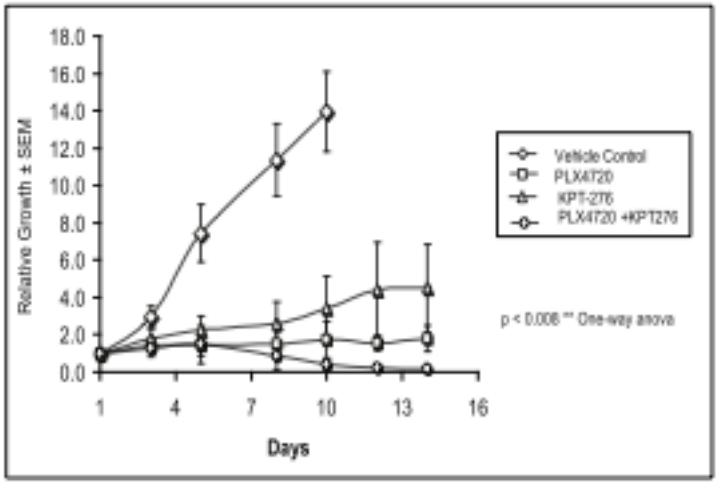



**Figure 8. Melanoma xenograft model : (A) BRAF V600 E mutant (B) NRAS mutant / BRAF wild-type (C) NRAS/BRAF wild-type.** (A) Melanoma xenograft using A375 cell line, treated with KPT-276, PLX4720 or the combination. Single therapy with either agent induces arrest of tumor growth while the combination of both agents induces complete regression of the tumors. For this model, KPT-276 75 mg/kg PO every other day, PLX4720 50 mg/kg i.p. daily or combination for 14 days were the treatment groups. (B) NRAS mutant / BRAF wild-type melanoma xenograft using Mel-Juso and (C) NRAS / BRAF wild-type melanoma xenograft using MeWo melanoma cell line were treated with KPT251 at 50 mg/kg PO 3 times per week for 21 days both xenograft models showed arrest in tumor growth.

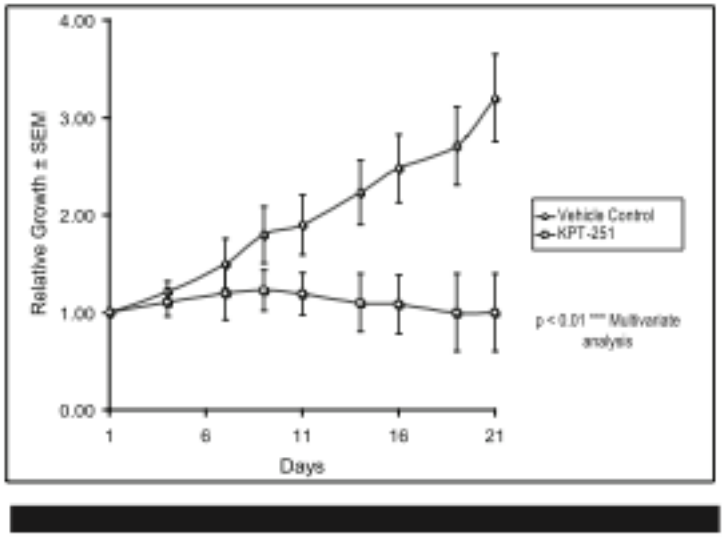
# A375




## NRAS WILD TYPE

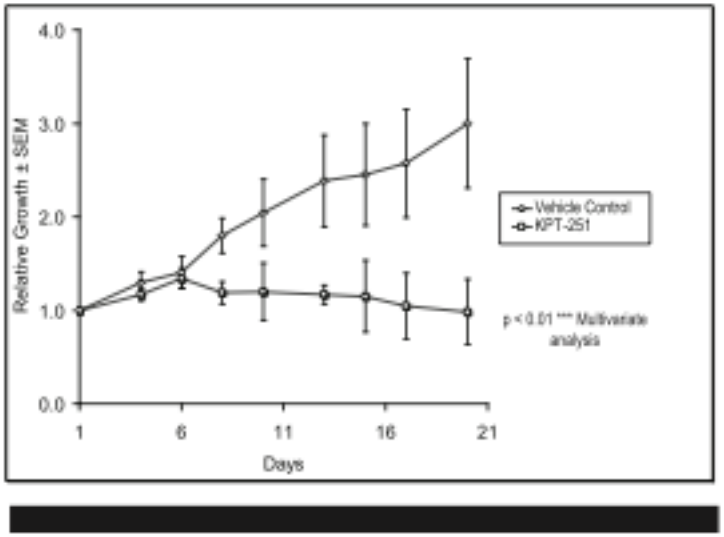
## BRAF MUTANT V600E

## MEWO




WILD TYPE BRAF / NRAS

## MEL-JUSO




NRAS MUTANT Q61L

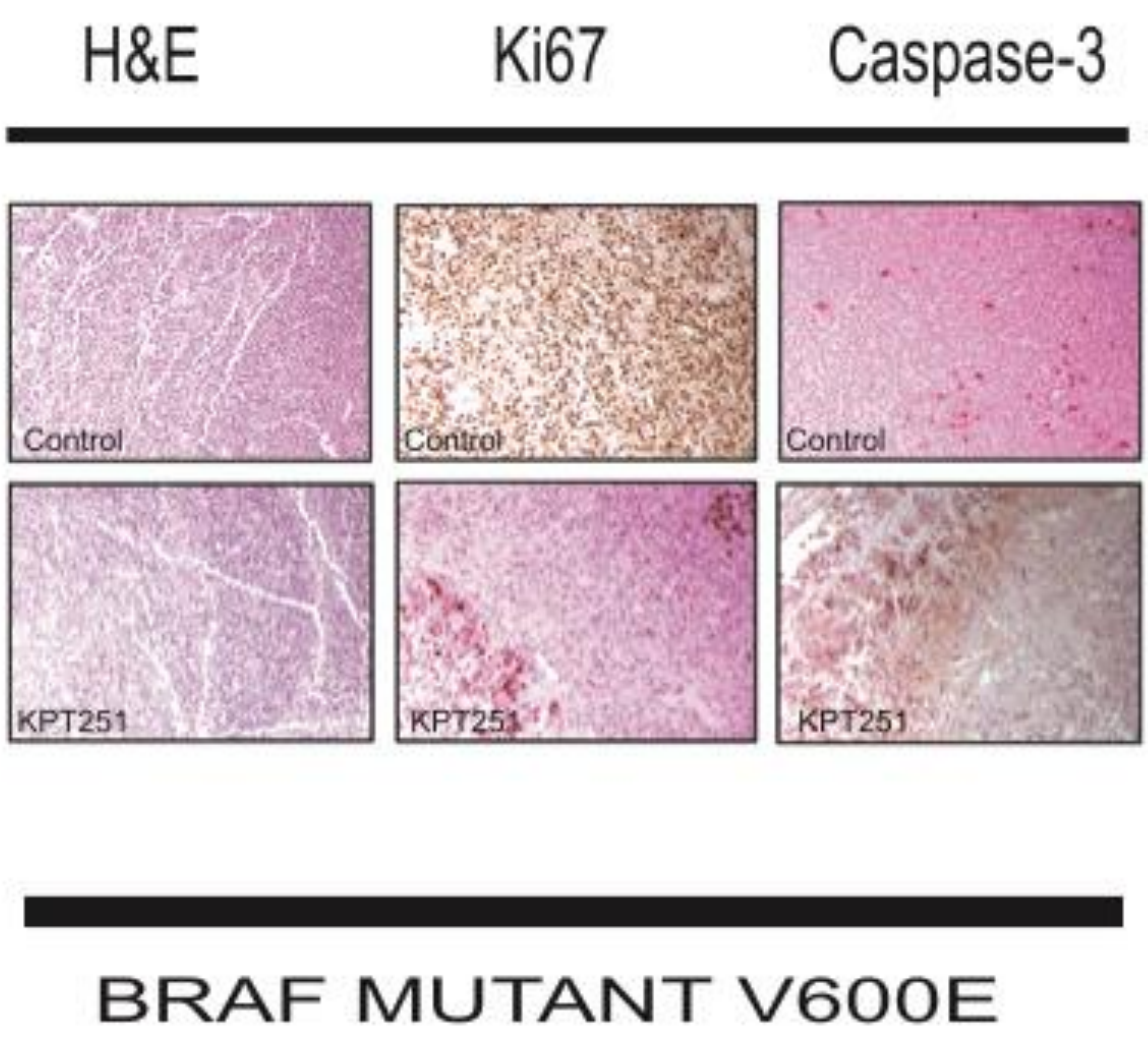



**Figure 9. Immunohistochemistry showing cell proliferation and apoptosis using Ki67 and cleaved Caspase-3 in excised tumors from our xenograft models.**

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## CONLUSION

For decades, nuclear export inhibition has shown to be an excellent target in tumor therapy. As single therapy for melanoma, CRM1 inhibition has shown potent antitumor activity in preclinical melanoma models independent of the mutational status of the tumor. The combination of a CRM1 inhibition with BRAF inhibition showed promising results in BRAF V600E mutant melanoma. Our results warrant further evaluation at a clinical level.