CRM1 and BRAF inhibition synergize and induce tumor regression in BRAF mutant melanoma.

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Running title: CRM1/BRAF inhibition in melanoma.

Keywords: CRM1, CRM1, BRAF, Melanoma
Disclosure of Potential Conflicts of Interest:

- Yosef Landesman, Jean Richard St Martin and Sharon Shacham are Karyopharm employees.
- Michael Kauffman has ownership interest in Karyopharm Therapeutics.
- Hensin Tsao is a consultant/advisory board member for Genentech, WorldCare Clinical and Quest.
- James C. Cusack received an unrestricted research grant from Karyopharm Therapeutics.

No other conflicts of interest to disclose.
Abstract

Resistance to BRAF inhibitor therapy places priority on developing BRAF inhibitor-based combinations that will overcome de novo resistance and prevent the emergence of acquired mechanisms of resistance. The CRM1 receptor mediates the nuclear export of critical proteins required for melanoma proliferation, survival and drug resistance. We hypothesize that by inhibiting CRM1-mediated nuclear export, we will alter the function of these proteins resulting in decreased melanoma viability and enhanced BRAF inhibitor anti-tumoral effects. To test our hypothesis, selective inhibitors of nuclear export (SINE) analogs KPT-185, KPT-251, KPT-276 and KPT-330 were used to induce CRM1 inhibition. Analogs PLX4720 and PLX4032 were used as BRAF inhibitors. Compounds were tested in xenograft and in-vitro melanoma models. In-vitro, we found CRM1 inhibition decreases melanoma cell proliferation independent of BRAF mutation status and synergistically enhances the effects of BRAF inhibition on BRAF-mutant melanoma by promoting cell cycle arrest and apoptosis. In melanoma xenograft models, CRM1 inhibition reduces tumor growth independent of BRAF or NRAS status and induces complete regression of BRAF V600E tumors when combined with BRAF inhibition. Mechanistic studies show that CRM1 inhibition was associated with p53 stabilization, and pRb and Survivin modulation. Furthermore we found that BRAF inhibition abrogates ERK phosphorylation associated with CRM1 inhibition, which may contribute to the synergy of the combination. In conclusion, CRM1 inhibition impairs melanoma survival in both BRAF mutant and WT melanoma. The combination of CRM1 and BRAF inhibition synergizes and induces melanoma regression in BRAF mutant melanoma.
Introduction

Most cancers acquire functional survival capabilities during their development. These capabilities include apoptosis evasion, self-sufficient growth signals, and insensitivity to anti-growth signals. Advanced-stage melanoma demonstrates these capabilities. For instance, a constitutively activate BRAF kinase is present in at least half of all advanced melanoma patients, driving melanoma proliferation. BRAF targeted therapy interrupts the growth signal and suppresses melanoma proliferation. Targeting BRAF mutation has led to outstanding clinical results with limited associated toxicity. However, the emergent resistance to BRAF inhibition limits the therapy’s response duration, challenging our limited therapeutic options for advanced melanoma.

The modulation of nucleo-cytoplasmic protein transport has been suggested as a possible therapeutic strategy in the treatment of cancer. Protein transport between the nucleus and the cytoplasm is critical for cell maintenance, cell proliferation and survival. Alterations in the expression of nuclear-transport-related proteins, in particular Exportin 1 (XPO1, also known as Chromosome Region 1, CRM1) are found in melanoma and other cancers. Overexpression of CRM1 is linked to inactivation of tumor suppressor proteins, apoptosis evasion and resistance to chemotherapy. In addition, CRM1 expression is of prognostic value in several types of cancer. CRM1 mediates nuclear export using nuclear export signals (NES), which are required for nuclear cargo transport from the nucleus to the cytoplasm. Selective inhibitors of nuclear export (SINE) also known as KPT-analogs (Figure 1; KPT-185, KPT-251, KPT-276 and KPT-330) are capable of binding to the Cys-528 residue in the cargo-binding portion of the CRM1 protein successfully preventing protein transport from the nucleus to the cytoplasm. The interruption of the system results in nuclear accumulation of the cargo, thereby restoring or disrupting cargo function. Among the CRM1 affected proteins, we find p42/44 MAPK (ERK1/2),
p53, c-Fos, Survivin and retinoblastoma protein (pRb). Modifying the function of these proteins alters survival and proliferation of cancer. Furthermore, the modulation of the CRM1 function using SINE has achieved promising preclinical results in cancer. Clinical grade analog, KPT-330, is currently in Clinical Phase I trials (NCT01607905 and NCT01607892).

CRM1 has been found to be overexpressed in metastatic melanoma when compared to nevi or even primary melanoma lesions. Moreover, CRM1 expression portrays independent of BRAF mutational status. This makes CRM1 a potential therapeutic target for metastatic melanoma. Furthermore, the concurrent presence of CRM1-overexpression and increased BRAF activity offers the possibility of using simultaneous CRM1 and BRAF inhibition to reduce melanoma survival in BRAF mutant melanoma. Therefore, we hypothesize that the inhibition of CRM1 in melanoma will result in impaired melanoma viability. In addition, by inhibiting independent targets using a CRM1/BRAF combination in BRAF mutant melanoma, we will have enhanced anti-tumoral effects which could translate in improved clinical outcomes.
Materials and Methods

Cell lines and reagents
Malignant melanoma cell lines where characterized and kindly donated by the Departments of Dermatology (Tsao lab) and Surgical Oncology (Wargo lab) from the Massachusetts General Hospital (MGH) between 2011 and 2012. Cells were authenticated following ATCC recommendations (ATCC Tech bulletin #8), and used within one week after authentication. Cells were passaged for less than six months after received. Cell morphology and growth analysis were performed posterior to resuscitation. Sequencing studies for PTEN, BRAF, NRAS and p53 among others have been performed in 2004, 2005, 2007, and in 2011 by Dr. Hensin Tsao (MGH) (supplementary methods table 1). All cell lines were maintained and all experiments were performed in DMEM (Sigma, D 6429) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Life Technologies). For in vitro studies, BRAF inhibitor PLX4032 (Selleck), and CRM1 inhibitor, KPT-251 or KPT-185 (Karyopharm) were used.

Cell Proliferation Assays
Cellular proliferation was evaluated by MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich) following manufacturer instructions. Cells were plated in 96-well plates at 1,000 to 10,000 cells per well in 100 μL of media 24 hours after treatment and MTT signal was read at 72 hours after treatment. The IC₅₀ and Combination Index (CI) by Chou-Talalay were determined from the regression plot logarithm of the concentration versus effect using CalcuSyn Software (Biosoft) v1.1. In addition, conservative isobolograms were used to show synergism and/or antagonism.
Immunoblotting, Immunofluorescence and Immunohistochemistry microscopy

Immunoblotting and immunofluorescence were performed using Cell Signaling general protocols. Immunohistochemistry was performed on paraffin-embedded tissue following IHC Products & Protocol Guide from R&D Systems. For our list of primary antibodies see supplementary methods table 2. Photomicrography images were recorded using a Nikon Eclipse 80i microscope and Qimaging Retiga Exi camber. Images were processed with Qimaging software (Version 2.1). For immunohistochemistry quantification, 3 mice per group were chosen at random 24 hours after first dose of the indicated agent. Quantification was done from 3 views chosen at random from the slides of the selected mice.

Cell cycle analysis by flow cytometry

Cell cycle analysis was carried out using Propidium iodide (PI) following manufacturer’s protocol in package insert (BD pharmigen). 3x10^4 cells per analysis were examined by flow cytometry (FACS calibur), and analyzed using WinMDI 2.9.

Caspase 3/7 activity assay

Caspase 3/7 activity was determined using Promega #G8091 system, following manufacturer’s protocol in package insert, and read using Victor3 multi-well reader (Wallac) and 1420 Wallac software.

Xenograft Model

Athymic nude mice Nu/Nu (Crl:NU-Foxn1nu), 4 weeks of age were purchased from Charles River Laboratories and Taconic Farms. All animal experiments were done in accordance with protocols approved by the MGH Subcommittee on Research Animal Care (SRAC#2011N000037). Cell lines A375, A2058 (BRAF V600E mutant) and Mel-Juso (BRAF...
WT and NRAS mutant) were injected SC at 5x10^6. MeWo (BRAF and NRAS WT) was injected SC at 1x10^7. Mice were randomized using a random number generator once an average of ~300 mm³ tumor volume was reached (For A375 and A2058, average time was 7 to 9 days; for Mel-Juso average time was 45 days and for MeWo average time was 82 days). After randomization, treatment was started. Mice were sacrificed following the guidelines by the Institutional Animal Care and Use Committee (IACUC) for MGH. Tumor volumes were determined using \[D \times (d^2)/2\], in which D represents the largest diameter of the tumor, and d represents the largest perpendicular volume to D. Tumor volumes were normalized individually to their initial volume (Volume at treatment day 1) (Relative tumor Volume = V_x/V_0, where V_x corresponds to the volume for the specific animal at a particular day and V_0 corresponds to the initial volume for the given animal. BRAF inhibitor, PLX4720 (Selleck), was diluted in DMSO to a 20mg/mL stock. Stock was diluted 1:10 in DMSO for daily i.p. injections. KPT251, KPT276 and KPT-330 were diluted in 0.6% w/v Pluronic F-68 and PVP K-29/32 solution to a 7.5 mg/mL stock suspension which was kept at room temperature, stirred, protected from light and used within 7 days. Statistical analysis consisted of Mann-Whitney Rank Sum Test, two-way ANOVA, and post hoc Bonferroni t test using GraphPad Prism, version 4.3. Oral administration of KPT-251 was tested to identify a dose and schedule that would yield maximum benefit with the least toxicity; we found 50mg/kg PO every other day as the best option. For combination studies we used: KPT-276 or KPT-330 PO 75 mg/kg or 10 mg/kg, respectively, every other day, PLX4720 i.p. or PO 25 - 50 mg/kg daily or their combination for 14 days. In experiments involving KPT-251 and KPT-330, all treatment groups contained 5 mice per group. For all experiments involving KPT-276, all treatment groups contained 10 mice per group. RECIST criteria were used to classify response, and are defined as follows: Complete Response (CR): Disappearance of all target lesions. Partial Response (PR): At least a 30% decrease in the sum of the longest diameter (LD) of target lesions, taking as reference the baseline sum LD. Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for
PD, taking as reference the smallest sum LD since the treatment started. Progressive Disease (PD): At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions.
Results and Discussion

CRM1 inhibition suppresses cell proliferation across a variety of cancer cell lines. Initially we screened the SINE compounds across a variety cancer cell lines, both solid and hematologic cancers. The interruption of nuclear export using novel SINE, KPT-185, induces inhibition of cell proliferation across a variety of cancer cell lines but not on the tested normal cell lines (Figure 2A). Cancer sensitivity to CRM1 inhibition in contrast to normal tissue may be explained by the altered expression of CRM1 and other nuclear transport proteins in cancer. Cancer sensitivity to CRM1 inhibition has led to the study of this target in several cancers with promising preclinical results.

CRM1 inhibition suppresses melanoma cell proliferation and is synergistic with BRAF inhibition, in-vitro. Melanoma BRAF mutant cell lines are sensitive to BRAF inhibition by PLX4032, which leads to inhibition of cell proliferation. However, BRAF WT melanoma cell lines are relatively resistant to BRAF inhibition (Figure 2B). CRM1 inhibition by SINE analogs results in inhibition of cell proliferation across tested melanoma cell lines. Among tested analogs, KPT-185 was almost twice as potent as KPT-251 or KPT-276 in-vitro. The results were independent of BRAF mutational status (Figure 2B and supplementary figure 1A-B). In other words, a BRAF mutation is required for BRAF treatment sensitivity but not for sensitivity to CRM1 inhibition. The combination of BRAF and CRM1 inhibition in BRAF mutant melanoma synergistically decreases cell proliferation in BRAF mutant melanoma (Figure 3A-B and Supplementary Figure 1C). Synergy between BRAF inhibition and CRM1 inhibition was maintained when testing different SINE analogs at different molar ratios (i.e. 1:1, 1:2 and 1:10) and different time points (24 to 96 hours) showing similar combination indexes. The greatest effect of the combination on cell proliferation was observed between 48 and 72 hours. Additionally, MEK/CRM1 combination, using MEK inhibitor U0126, results in similar synergy as the BRAF/CRM1 combination in BRAF
mutant melanoma (data not shown). Furthermore, MEK/BRAF/CRM1 combination further shifts the IC50 in BRAF mutant melanoma and results in synergism for the three drug combination. These findings might suggest a MAPK pathway component as a responsible for the observed synergy. For the BRAF WT cell lines, the combination index for the BRAF/CRM1 combination varied according to drug dose and cell line and ranged from antagonistic to synergy (Supplementary Figure 1C).

**CRM1 inhibition induces cell cycle arrest and apoptosis, and synergistically increases cell death when combined with BRAF inhibition.** XPO1/CRM1 inhibition is associated with cell cycle arrest. In melanoma, SINE progressively reduce S-phase in both BRAF mutant and WT cell lines, inducing cell cycle arrest 24 hours after treatment (Supplementary Figure 2A). Both G1 and/or G2 cell cycle arrest can be observed using the CRM1 inhibitors (Supplementary Figure 2B). During the next 48 hours, an increase in the sub-G1 population becomes statistically significant peaking at 72 hours of CRM1 inhibition. BRAF inhibition leads to a predominant G1 cell cycle arrest in BRAF mutant melanoma cell lines with a dose-cell line dependent increase in the sub-G1 population (Supplementary Figure 2C-D and 3A-D). We observed no statistically significant effect on the cell cycle after BRAF inhibition in the BRAF WT cell lines at tested doses (Supplementary Figure 3C-D). The combination of both compounds induced a statistically significant greater increase in the sub-G1 population in BRAF mutant cell lines in comparison to either single therapy at tested doses (p < 0.05) (Supplementary Figure 2B-D and 3A-D). There was a significant (p < 0.05) increase in the sub-G1 population of the BRAF WT cell lines after drug combination at higher doses (> 3μM) when compared to single therapy with KPT (Supplementary Figure 3) but no other statistically significant changes in the cell cycle. This can be attributed to reaching doses close to BRAF WT, BRAF inhibition IC50s.
We used caspase-3/7 activity as an apoptosis surrogate. Both CRM1 and BRAF inhibition increase caspase-3 and 7 activity in the tested melanoma cell lines in a dose and time related manner (p < 0.05) (Supplementary Figure 4). A modest effect of BRAF inhibition on caspase activity in some of the BRAF mutant melanoma cell lines suggests cell cycle arrest over apoptosis as the predominant mechanism impacting cell proliferation in these cell lines, under the tested conditions. These findings correlate to the results from the cell cycle experiments.

The combination of both compounds results in a statistically significant increase in caspase activity when compared to either single therapy in BRAF mutant melanoma. This would correlate to strong synergy (CI < 0.3) for caspase-3/7 activity on BRAF mutant cell lines if the combination was taken as maximal effect and Chou-Talalay is applied. Despite slight increase in caspase activity after BRAF inhibition in BRAF WT cell lines after single drug treatment, the combination resulted in no statistically significant increase on caspase activity at tested doses when compared to CRM1 inhibition alone. For either single drug or combination studies, the effect on caspase activity was time- and dose-dependent and correlated to the sub-G1 changes observed in the cell cycle.

In melanoma xenograft models, CRM1 inhibition suppresses tumor growth, and induces complete regression of A375 Melanoma BRAF V600E tumors when combined with BRAF inhibition.

Oral administration of KPT-251 suppressed tumor growth in three xenograft models (Supplementary Figure 5A). Tumor growth suppression was associated with increased cleaved caspase-3 staining and a decreased Ki67 staining (Supplementary Figure 5B). These findings suggest increased apoptosis, in addition to decreased cell proliferation. Similar to the in-vitro data, the effects of CRM1 inhibition were independent of BRAF and NRAS status.
For our combination studies, BRAF inhibitor, PLX-4720 (preclinical PLX-4032 analog), was used due to better bioavailability. CRM1 inhibitors KPT-276 and KPT-330, structural analogs of KPT-251 with better bioavailability, were used to test BRAF and CRM1 combination in our BRAF V600E melanoma xenograft model. Both KPT-276 and PLX-4720 decreased tumor growth as single therapy. The combination of both inhibitors induced complete tumor regression per RECIST criteria (Figure 4A, Red Line) and the difference between both single therapy and the combination therapy was statistically significant. These findings correlated to decreased cell proliferation and increased apoptosis when compared to the control group (Figure 4B-C). The effect on proliferation is not statistically different between treatment groups. We believe the greatest contribution of the combination is the effect on apoptosis, which is significantly increased (p < 0.05) by the drug combination. Decreased cell proliferation complemented by increased apoptosis explains the observed tumor regression. Treatment was stopped after 14 days, after which tumor growth was observed in all treatment groups. Survival for the combination group was statistically longer than for either single therapy. As single therapy and in combination, both inhibitors were well tolerated with no significant effect on animal weight. Similar results were observed using clinical grade analog KPT-330 in A2058, PTEN null / BRAF mutant melanoma cell line using a different treatment schedule (Supplementary Figure 6A-B).

**CRM1 inhibition modulates levels of p53, pRb, Survivin and ERK phosphorylation.**

TP53 is a known tumor suppressor capable of inducing cell cycle arrest and apoptosis. Mutations in p53 disable its tumor suppressor abilities. While mutations in p53 are not common in melanoma patients (90% of melanoma tumors are p53 wild-type), loss of p53 tumor suppressor function is lost in many melanoma tumors owed to either, mutations in CDKN2A and/or overexpression of HDM2. The alteration of these p53 regulators facilitate CRM1-mediated nuclear export of p53 and cytoplasmic degradation of p53, thereby rendering the cells...
functionally deficient for the tumor suppressor p53. The inhibition of nuclear export using the CRM1 inhibitor favors nuclear localization of p53 and prevents cytoplasmic p53 degradation (Figure 5A-B). The stabilization of p53 by CRM1 is independent of DNA damage (Figure 5C) and is associated with increased levels of p53 targets (i.e. p21 and HDM2) in p53 WT melanoma but not in p53 mutant cell lines. TP53 knockdown induced partial reduction of CRM1 effects on cell proliferation (Figure 5D) but not on caspase activity as single therapy or in the combination with BRAF inhibition (Supplementary Figure 7). We can suggest that p53 has at least a partial role in the CRM1-mediated anti-tumoral effects. However, we must propose the existence of p53-independent mechanism(s) which contribute to the CRM1 p53-mediated effects. These alternative mechanisms would account for the remaining observed anti-tumoral activity and the resulting synergy of the combination. In addition, p53 nuclear localization is consistent after CRM1 inhibition and may serve as a marker of successful CRM1 inhibition.

Other proteins we found to be affected by CRM1 inhibition include tumor suppressor retinoblastoma protein, Survivin and ERK. Retinoblastoma protein (pRb) and its phosphorylation are both affected by CRM1 and BRAF inhibition. The combination of both agents further reduces pRb and p-pRb levels (Figure 5E). Hypo-phosphorylated pRb blocks proliferation by preventing the transcription of genes essential for cell cycle progression. These finding correlate to our cell cycle analysis suggesting a role for pRB in CRM1 mediated cell cycle arrest.

Both CRM1 and BRAF inhibition decrease Survivin levels. This finding is also seen after the drug combination (Figure 5E). Survivin, also called baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), is known as a bi functional protein for its role in cell division and apoptosis suppression. Survivin is constantly shuttling between the nucleus and the cytoplasm. Cytoplasmic localization of Survivin is associated with anti-apoptotic function, while its nuclear
pool mediates its mitotic function. However, CRM1 inhibition disrupts the cell division and anti-apoptotic functions of Survivin.

CRM1 inhibition increases ERK phosphorylation in both BRAF WT and Mutant (Figure 5E) (6). ERK phosphorylation after treatment with anti-tumoral agents has been described as a pro-survival event. Thus, phosphorylation of ERK following CRM1 inhibition could translate into enhanced chemo-resistance. Another tentative explanation would involve ERK nuclear localization as an anti-proliferative factor and further explaining CRM1-related anti-proliferative effects. Interestingly, the combination of the CRM1 inhibitor with the BRAF inhibitor prevents ERK phosphorylation (Figure 5E) and results in synergy between the two compounds. Our data suggest that the protection against CRM1-induced ERK phosphorylation by BRAF-inhibition may play a role in the synergistic response to combined therapy. Furthermore, our data supports that the inhibition of ERK phosphorylation may have a role in addressing ERK-mediated chemo-resistance.

In conclusion, given the discovery of diverse mechanisms of acquired resistance to BRAF inhibitor therapy which are not readily countered with pharmacologic strategies, we believe a priority should be placed on developing BRAF inhibitor-based combinations to overcome de novo resistance and prevent the emergence of these acquired resistance mechanisms. We believe achieving this goal would translate into longer duration of response (Progression Free Survival) and will increase the percentage of treatment responders. Based on our data, CRM1/BRAF inhibitor combination can offer a benefit to the melanoma patient population. The combination of CRM1 and BRAF inhibition results in a synergistic decrease of cell proliferation, and increased apoptosis in BRAF mutant melanoma cell lines and xenograft models. We attribute the synergy of the combination to the inhibition of independent targets altering multiple essential factors of melanoma viability. In particular, we find that the abrogation of the CRM1-
associated ERK phosphorylation by BRAF inhibition to be one of the contributors. Taken together, our findings offer rationale for the combination of CRM1 and BRAF inhibition in an attempt to improve the treatment of patients with BRAF mutant melanoma. Furthermore, the effects of CRM1 inhibition as single therapy are independent of BRAF status. This suggests a role for CRM1 inhibition in the treatment of melanoma, without mutational limitations. Studies exploring the safety and efficacy of CRM1 and CRM1/BRAF inhibition in the melanoma clinical realm are needed.
References


Figure Legends

Figure 1. Chemical structures for selective inhibitors of nuclear export (SINE) analogs KPT-185, KPT-251, KPT-276 and KPT-330.

Figure 2. Box plots showing cell proliferation IC50s after XPO1 inhibition. (A) IC50 doses for cell proliferation after XPO1 inhibition with KPT-185 across a panel of normal and cancer cell lines. (B) Comparison of BRAF inhibitor, PLX4032, and XPO1 inhibitor, KPT-185, IC50 concentrations in melanoma cell lines, sorted by BRAF status. Error bars represent standard deviation; "n" represent number of cell lines tested.

Figure 3. Nuclear export inhibition decreases melanoma cell proliferation and is synergistic with BRAF inhibition in vitro. (A) MTT assay demonstrating the effect of XPO1 inhibition, BRAF inhibition, and their combination, on cell proliferation with their respective combination index (CI) value. (B) Corresponding conservative isobologram for the MTT assay shown on (A). Drug combinations at 1:1 ratio. Error bars represent standard deviation.

Figure 4. In BRAF mutant xenograft models, nuclear export inhibition induces delayed tumor growth as single therapy, and tumor regression when combined with BRAF inhibition. (A) Relative tumor volumes curves for melanoma A375 xenografts after treatment with KPT-276 (XPO1 Inhibitor) at 75 mg/kg every other day, PLX-4720 (BRAF inhibitor) at 50 mg/kg ip every day or their combination for 14 days. (B-C) Immunohistochemistry demonstrating Ki67 and Cleaved Caspase-3 staining in harvested tumor samples and the quantification of the staining. Panel A: Each treatment group consisted of 10 mice. Error bars represent SEM. In panel B, error bars represent STD. In panel C, Bar represent 200 μm. ** Denotes p <0.01; n.s. denotes not statistically significant (p > 0.05). † denotes control group reached tumor volume end of study threshold.
Figure 5. Nuclear export inhibition modulates levels of TP53, pRb, p-ERK and Survivin

(A) Immunofluorescence and (B) Western blot time course from whole and fragmented cell lysate demonstrating nuclear localization and stabilization of p53 after XPO1 inhibition using KPT-251 (1μM).

(C) Western blot from nuclear lysate demonstrating the effect of XPO1 inhibition (KPT-251; 1μM) on DNA damage markers. Etoposide (25μM) was used as a positive control for DNA damage. (D) MTT assay demonstrating the effect of XPO1 inhibition (KPT-251) on cell proliferation after p53 knockdown, in A375 melanoma cell line. (E) Western blot demonstrating the effect of XPO1 inhibition, BRAF inhibition and their combination, on PARP, Survivin, pRb and ERK in A375 melanoma.
Figure 1

KPT-185

KPT-276

KPT-251

KPT-330
A. IC50 (M) (n=5) (n=17) (n=13) (n=8) (n=5) (n=34) (n=9) (n=7) (n=10)

B. IC50 (M) (n=10) (n=3) (n=2)
Figure 3
**Figure 4**

A. Absolute tumor volume ($x10^3 \text{mm}^3$) over 14 days of treatment for Vehicle (n=10), PLX4720 (n=10), KPT-276 (n=10), and PLX+KPT (n=10) groups.

B. Ki67 Staining (%) and Caspase-3 Staining (%) for PLX-4720 and KPT-276 treatments.

C. Ki67 and Caspase-3 staining images for Tumor #1, Tumor #2, and Tumor #3 in Vehicle, PLX4720, KPT-276, and PLX+KPT conditions.

**Note:** Images show representative staining patterns with scale bars included for comparison.
Figure 5
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Mol Cancer Ther Published OnlineFirst April 24, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-12-1171

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