Induction of p53-mediated transcription and apoptosis by exportin-1 (XPO1) inhibition in mantle cell lymphoma

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Mantle cell lymphoma (MCL) is an incurable form of B-cell, non-Hodgkin’s lymphoma (NHL). Given this grievous outcome, novel, effective treatments are urgently needed for this disease. The main therapeutic challenge in MCL is the implementation of treatment strategies that maximize the efficient induction of lymphoma cell apoptosis without the development of chemoresistant sub-clones.

The nuclear–cytoplasmic transport of proteins and ribonucleic acids is vital to cellular homeostasis in eukaryotic cells. This process is regulated, in part, by the karyopherin-β protein family. The XPO1 protein (exportin-1, also known as CRM1), is among seven exportins. Interestingly, XPO1 is the only exportin that mediates the transport of numerous proteins including tumor suppressor, growth regulatory, and anti-apoptotic proteins as well as several mRNAs and ribosomal proteins that are essential for ribosomal biogenesis. Exportin-1 is abnormally highly expressed/upregulated in a variety of solid tumor types, as well as hematological malignancies including MCL. In fact, the overexpression of XPO1 is positively correlated with poor disease prognosis in some malignancies. Therefore, it has been suggested that alterations in nuclear–cytoplasmic trafficking, and hence the aberrant cytoplasmic localization, of tumor suppressor proteins, cell cycle regulators, and/or pro-apoptotic proteins, as well as the deregulation of ribosomal biogenesis, can cause oncogenesis and the development of resistance to chemotherapeutic agents.

More than 90% of MCL patients have extranodal manifestations, including circulating lymphoma cells, bone marrow, and gastrointestinal tract involvement. TP53 mutations occur in 15–20% of the cases of MCL, and wild-type p53 is inactivated by upstream gene amplification of BMI1 (~10%), homozygous deletion of CDKN2A (INK4a/ARF) (15–20%), the overexpression of human homolog of murine double minute 2 (MDM2) (~5%), or TP53 gene deletion (25–30%). All of the nuclear transporter exportin-1 (XPO1) is highly expressed in mantle cell lymphoma (MCL) cells, and is believed to be associated with the pathogenesis of this disease. XPO1-selective inhibitors of nuclear export (SINE) compounds have been shown to induce apoptosis in MCL cells. Given that p53 is a cargo protein of XPO1, we sought to determine the significance of p53 activation through XPO1 inhibition in SINE-induced apoptosis of MCL cells. We investigated the prognostic impact of XPO1 expression in MCL cells using Oncomine analysis. The significance of p53 mutational/functional status on sensitivity to XPO1 inhibition in cell models and primary MCL samples, and the functional role of p53-mediated apoptosis signaling, were also examined. Increased XPO1 expression was associated with poor prognosis in MCL patients. The XPO1 inhibitor KPT-185 induced apoptosis in MCL cells through p53-dependent and -independent mechanisms, and p53 status was a critical determinant of its apoptosis induction. The KPT-185-induced, p53-mediated apoptosis in the MCL cells occurred in a transcription-dependent manner. Exportin-1 appears to influence patient survival in MCL, and the SINE XPO1 antagonist KPT-185 effectively activates p53-mediated transcription and apoptosis, which would provide a novel strategy for the therapy of MCL.
these abnormalities essentially lead to the loss of p53 tumor suppressor activity.

The nuclear export of p53 is cooperatively mediated by MDM2 and XPO1.\(^{(17)}\) MDM2 activates the nuclear export signal (NES) in p53 through its E3 ubiquitin ligase activity, leading to a conformational change in p53 that exposes p53’s NES domain. Following ubiquitination, XPO1 recognizes p53’s NES and exports the protein from the nucleus to the cytoplasm, where it is unable to execute transcriptional activity to regulate cell fate. As we mentioned previously, XPO1 is highly expressed in MCL cells,\(^{(8)}\) which may limit p53-mediated transcriptional activity, and hence the ability of p53 to trigger apoptosis.\(^{(18)}\) It has been reported that wild-type p53 is abnormally sequestered in the cytoplasm in certain human tumor cells.\(^{(19,20)}\)

Novel small-molecule, drug-like, potent, and covalent XPO1-selective inhibitors of nuclear export (SINE) compounds were recently developed. These compounds selectively bind to the Cys528 of XPO1, thereby inhibiting XPO1 binding to the NES domains of its cargo protein.\(^{(21)}\) The SINE KPT-185 has been shown to induce apoptosis in MCL cells.\(^{(8)}\) The inhibition of XPO1 is believed to maintain the nuclear localization, and hence function, of p53.\(^{(1,3)}\) Furthermore, XPO1 is involved in the nuclear export of numerous proteins including p21, p27, p73, nucleophosmin-1, PP2A, FOXO, β-catenin, APC, topoisomerase II, and 1kb \(^{(1)}\) This would suggest that the biological significance of p53 activation in XPO1 inhibition-induced apoptosis in MCL cells is highly unspecified and thus in need of further elucidation. Accordingly, we examined the pathological significance of XPO1’s influence on p53 cellular localization and functional activity and its potential as a therapeutic target for enhancing MCL cell apoptosis.

Materials and Methods

Reagents. The selective XPO1 inhibitor KPT-185 was synthesized and provided by Karyopharm (Karyopharm, Natick, MA, USA). The selective small-molecule antagonist of MDM2, Nutlin-3a was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cells and cell culture. A total of 16 lymphoid cell lines, including six MCL cell lines, were cultured in RPMI-1640 medium containing 20% heat-inactivated FBS (Table 1). Z-138 and JVM-2 have wild-type p53, whereas MINO, JeKo-1, MAVER-1, and NCEB-1 have defective (i.e., missense mutated or deleted) p53.\(^{(2,22)}\) The Z-138 and JVM-2 cells were transduced with retroviruses encoding either p53-specific shRNA (nucleotides 611–629, Genbank NM000546) or scrambled shRNA and stable shRNA-expressing cells were generated.\(^{(23)}\) The cell were harvested in log-phase growth, seeded at a density of 1\(\times\)10\(^5\) cells/mL, and were exposed to the indicated compounds.

Annexin V positivity, relative to the cell lines’ p53 mutational status

| Cell line | KPT-185, nM | Nutlin-3a, μM | p53 Status |
|-----------|-------------|---------------|------------|
| Z-138†    | 85          | 1.1           | Wild-type  |
| JVM-2†    | 641         | 5.9           | Wild-type  |
| REH       | 371         | 6.1           | Wild-type  |
| NALM-6    | 32          | 2.1           | Wild-type  |
| RS4;11    | 198         | 2.4           | Wild-type  |
| MT-2       | 49          | 5.0           | Wild-type  |
| TMD8      | 54          | 1.4           | Wild-type  |
| SUP-M2    | 81          | 1.4           | Wild-type  |
| MINO†     | 1865        | >10           | Mutant (V147G) |
| JeKo-1†   | 703         | >10           | Mutant (deletion) |
| MAVER-1†  | 391         | >10           | Mutant (D281E) |
| NCEB-1‡   | 3518        | >10           | Mutant (E221K) |
| Raji       | 418         | >10           | Mutant (R213Q, Y234H) |
| Jurkat     | 1285        | >10           | Mutant (R196V) |
| ATM-1      | 279         | >10           | Mutant (V272L) |
| SU-DHL-1   | 185         | >10           | Mutant (R273H) |

†Mantle cell lymphoma cell lines.

Heparinized peripheral blood and pleural effusion samples were obtained from MCL patients after informed consent, according to the University of Texas MD Anderson Cancer Center (Houston, TX, USA) guidelines in accordance with the Declaration of Helsinki. Mononuclear cells were purified by density-gradient centrifugation, and non-adherent cells were resuspended at a density of 1\(\times\)10\(^6\) cells/mL. Cell viability was evaluated by triplicate counts of Trypan blue dye-excluding cells.

Apoptosis analysis. Evaluation of apoptosis by the annexin V-propidium iodide binding assay was carried out as described previously.\(^{(11)}\) Apoptosis was quantified as the percentage of annexin V-positive cells, and the percent of drug-specific apoptosis was assessed by the formula: (% test – % control) \(\times\) 100/(100-% control).

Western blot analysis and co-immunoprecipitation. Western blot analysis was carried out as described previously.\(^{(24)}\) The following antibodies were used: mouse monoclonal anti-p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit polyclonal anti-XPO1 (Santa Cruz Biotechnology); rabbit polyclonal anti-HSP-90α/b (Santa Cruz Biotechnology); rabbit monoclonal histone H3 (Cell Signaling Technologies, Beverly, MA, USA); rabbit monoclonal GAPDH (Cell Signaling Technologies); and mouse monoclonal anti-β-actin (Sigma Chemical Co., St Louis, MO, USA). Nuclear and cytoplasmic proteins were extracted using a subcellular fractionation kit (ProteoExtract; EMD Millipore, Billerica, MA, USA), according to the manufacturer’s protocol. Protein lysates were also subjected to immunoprecipitation using anti-XPO1 and immunoprecipitates were subjected to Western blot analysis with anti-XPO1 or p53. Visualized blots were analyzed by the Multi Gauge 3.1 software (Fujifilm, Tokyo, Japan).

Gene expression analysis. The mRNA expression levels were quantified using TaqMan gene expression assays (TNRFSF10B (DR5), Hs00366272_m1; FAS, Hs00163653_m1; BRC3 (PUMA), Hs00248075_m1; TP53INP1, Hs00264502_m1; GAPDH, Hs99999905_m1; Applied Biosystems, Foster City, CA, USA) on a 7900HT Fast Real-Time PCR System.\(^{(25)}\) Relative quantification between different samples was determined according to the 2\(^{-\Delta\Delta C_{\text{t}}}\) method with a relative quantification (RQ)\(_{\text{min}}\)/RQ\(_{\text{max}}\) confidence set at 95%, using SDS RQ Man-

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agnostic impact on MCL overall disease survival, and, as such, results suggest that XPO1 overexpression has a negative prognosis.

Overexpression of XPO1 associated with poor disease prognosis in MCL patients. The mRNA expression levels in MCL patient samples were determined using Oncomine data (Compendia Bioscience, Ann Arbor, MI, USA). Our gene expression analyses showed an increase in XPO1 mRNA expression in the MCL samples (n = 8) versus the normal B-cell controls (n = 5) (P < 0.001; GSE2350). In fact, higher XPO1 expression was associated with a poorer prognosis in MCL patients (i.e., a median overall survival of 3.2 years in the low expression XPO1 cases vs 1.9 years in the high expression XPO1 cases, P = 0.033) (Fig. 1a). Patients who survived for 5 years or more with MCL had lower levels of XPO1 mRNA (P = 0.004) (Fig. 1b). In contrast to XPO1, the differential expression of MDM2 did not show clinical significance in MCL patients. The MDM2 expression levels were not statistically significantly higher in the MCL samples (n = 8) compared to normal B-cell controls (n = 5) (P = 0.27; GSE2350), and the levels of MDM2 were not associated with overall disease survival of these patients (P = 0.12 at 5 years, n = 63). These results suggest that XPO1 overexpression has a negative prognostic impact on MCL overall disease survival, and, as such, XPO1 is potentially a therapeutic target in this disease.

Mutational status of p53 affects lymphoid cell sensitivity to KPT-185. Fifteen to 20% of MCL cases have deleterious p53 mutations (13) and it has been recently reported that KPT-185 induces apoptosis in MCL cells (8) Thus, we investigated the possibility that the p53 mutational status of MCL cells could affect their sensitivity to XPO1 inhibition by KPT-185. We determined ED50 values for phosphatidylserine externalization (i.e., the effective dose-induced apoptotic cell killing in ~50% of the sample population as measured by annexin V positivity) in 16 lymphoid cell lines with known p53 mutational status. As shown in Table 1, the p53 mutational status was generally associated with a decrease in sensitivity to KPT-185.

As a positive control for wild-type p53-mediated apoptosis induction, we used the selective MDM2 inhibitor Nutlin-3a (26,27). As shown in Table 1, Nutlin-3a, like KPT-185, had decreased efficacy in the p53-mutated MCL cells. The mutant p53 cell lines were significantly less sensitive to KPT-185 than those with wild-type p53 (P < 0.01, Fig 2a). Next, we correlated the extent of apoptosis induced by KPT-185 with that induced by Nutlin-3a, specifically in eight of the p53 wild-type cell lines, as a potential cellular outcome of wild-type p53 activation. The extent of apoptosis induced by KPT-185 significantly, positively correlated (i.e., P = 0.04, r = 0.73, Fig. 2b) with that induced by Nutlin-3a, implying that KPT-185 could utilize p53 signaling to induce apoptosis in lymphoid cells.

We wanted to determine if wild-type p53 expression levels could determine susceptibility of the MCL cells to XPO1 inhibition. To this end, p53 wild-type Z-138 and JVM-2 MCL cells were transduced with lentivirus encoding either negative control shRNA or p53-specific shRNA, and stable shRNA-expressing cells were generated. The p53-specific shRNA reduced p53 levels by 80–90%, and these p53 knockdown cells were also significantly less sensitive to both Nutlin-3a- and KPT-185-induced apoptosis (i.e., P < 0.05 in drug-specific annexin V induction at all concentrations examined; Fig. 2c), suggesting that both the MDM2 inhibitor Nutlin-3a and the XPO1 inhibitor KPT-185 activate p53-mediated signaling to induce apoptosis in MCL cells.

To further define the observed wild-type p53-dependent apoptosis induced by KPT-185, we used isogenic B-lymphoma cells from Eμ-TCL1 transgenic mice with different p53 backgrounds. As expected, the lymphoma cells derived from the TCL1-Tg:p53R172H/R172H mice were markedly less sensitive to Nutlin-3a than those derived from the TCL1-Tg:p53WT/WT mice (Fig. 2d). The presence of p53R172H also limited the apoptotic activity of KPT-185. However, higher concentrations of KPT-185 (400 nM) were able to induce some extent of apoptosis in lymphoma cells from the TCL1-Tg:p53R172H/R172H mice, indicating a p53-independent effect of KPT-185. Taken together, data suggest that the functional status of p53 is a determinant of XPO1 inhibition-induced apoptosis in lymphoid cells.

KPT-185 increases nuclear p53 levels and activates p53-mediated transcription in MCL cells. Next, we investigated the possible mechanistic differences between Nutlin-3a- and KPT-185-induced apoptosis. The primary function of p53 is as a transcription factor to induce p53 targets and apoptosis, which
occurs in the nucleus, although in some circumstances cytoplasmic p53 may trigger apoptosis in a transcription-independent manner.\(^{18,29}\) To determine localization of induced p53, Z-138 MCL cells were treated with Nutlin-3a or KPT-185 and were subjected to subcellular fractionation, and the levels of p53 in the nuclear and cytoplasmic fractions were determined by immunoblotting. In contrast to Nutlin-3a, which increased p53 levels both in the cytoplasm and the nucleus, KPT-185 accumulated p53 only in the nucleus (Fig. 3a,b). KPT-185 treatment resulted in a reduced interaction between XPO1 and

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**Fig. 2.** Status of p53 signaling determines exportin-1 (XPO1) inhibition-induced apoptosis. (a) ED\(_{50}\) values for annexin V induction (effective dose inducing 50% cell killing as measured by annexin V positivity) of 72-h exposure to KPT-185 were determined in 16 lymphoid cell lines with known p53 mutational status (eight cell lines with wild-type p53 [WT] and eight with mutant p53 [MUT]), and the values were compared between p53 wild-type and mutant cells. Average values were expressed as mean ± SEM. (b) ED\(_{50}\) values for annexin V induction of 72-h exposure to KPT-185 were correlated with those to Nutlin-3a in p53 wild-type cell lines. (c) Levels of p53 determined sensitivity to MDM2/XPO1 inhibition in p53 wild-type MCL cells. Transduced Z-138 and JVM-2 cells (virus encoding either negative control shRNA [shC] or p53-specific shRNA [shp53]) were incubated with the indicated concentrations of KPT-185 or Nutlin-3a for 72 h, and the annexin V-positive fractions were measured by flow cytometry. Results are expressed as mean ± SD of triplicate measurements. Intensity of the p53 immunoblot signals relative to that of β-actin was calculated, and the levels in shC cells were set as 1.0. (d) Mutational status of p53 affects lymphoma cell sensitivity to Nutlin-3a and KPT-185 in an Eμ-TCL1 mouse model. Eμ-TCL1 mice spontaneously develop B-lineage lymphomas. Cells were collected from affected spleens of TCL1-Tg:p53\(^{WT/WT}\) mice (WT; \(n = 9\)) and TCL1-Tg:p53\(^{WT/R172H}\) mice (MUT; \(n = 6\)), and they were exposed to KPT-185 (100 nM or 400 nM) or Nutlin-3a (10 μM) for 72 h. The annexin V-positive fractions were measured by flow cytometry. Average values were expressed as mean ± SEM.
p53 (Fig. 3c), indicating that KPT-185 disrupts XPO1–p53 interaction.\textsuperscript{36} These results suggest that Nutlin-3a treatment causes an increase in the total levels of cellular p53, whereas KPT-185 treatment induces nuclear p53 by disrupting the XPO1–p53 interaction. To determine the activation potential for p53-mediated transcription in MCL, Z-138 and JVM-2 cells were treated with KPT-185 or Nutlin-3a at, or twice, their ED\textsubscript{50} concentration (Table 1), and the transcriptional activation of p53-regulated target genes were assessed. As shown in Figure 4, only KPT-185 induced the expression of p53 target genes in Z-138 cells, indicating different effector pathways for p53-induced apoptosis in these cells. In JVM-2 cells, both KPT-185 and Nutlin-3a activated p53 target genes. The data suggest that KPT-185 primarily activates p53-mediated transcription to induce apoptosis. To elucidate this, Z-138 cells expressing p53-specific or scrambled shRNA were exposed to KPT-185 at 2 \times \text{ED}_{50} concentration, and KPT-induced DR5 and FAS transcripts compared to untreated cells were determined. KPT-185 induced significantly less DR5 and FAS in p53 knockdown Z-138 cells than Z-138 cells expressing scrambled shRNA (3.20 \pm 0.50 vs. 1.75 \pm 0.32-fold increase in DR5, \(P = 0.01); and 3.40 \pm 0.19 vs. 0.63 \pm 0.12-fold increase in FAS, \(P < 0.0001). However, although MDM2 contributes to p53 nuclear export, Nutlin-3a may not always activate p53-mediated transcription, probably in a cell-specific manner. Cytoplasmic retention of Nutlin-induced p53 has been reported in neoplastic cell lines and patient cells.\textsuperscript{31–34} Neither KPT-185 nor Nutlin-3a altered p53 mRNA levels (data not shown), suggesting that they have little effect on p53 synthesis.

Fig. 3. Exportin-1 (XPO1) inhibitor KPT-185 increases nuclear p53 levels. (a) Z-138 mantle cell lymphoma cells were treated with indicated concentrations of Nutlin-3a (N3a) or KPT-185 (KPT) for 14 h and subjected to subcellular fractionation. The levels of p53 in the cytoplasmic and nuclear fractions were determined by immunoblotting. The relative purity of the cytoplasmic and nuclear fractions was respectively determined by sequential probing for the cytoplasmic marker HSP90 and the nuclear marker histone H3. The relative intensity of p53 compared to HSP90 (cytoplasm) or histone H3 (nucleus) was calculated, and the value in untreated cells (C) was set as 1. Results are expressed as mean \pm SD of triplicate experiments. *Significance at \(P < 0.05). (b) p53 expression in whole cell lysate (WCL) of Z-138 cells, which were treated with indicated concentrations of Nutlin-3a or KPT-185 for 14 h. The relative intensity of p53 compared to GAPDH was calculated, and the value in untreated cells (C) was set as 1. Results are representative of three independent experiments. (c) KPT-185 disrupts CRM1–p53 interaction. Z-138 cells were treated for 1 h with 160 nM KPT-185. Exportin-1 was immunoprecipitated (IP) from total cell lysates and its association with p53 was determined by Western blot analysis. The relative p53 expression levels were normalized against XPO1 in which untreated Z-138 cells (C) served as 1. IgG, heavy chain IgG.

KPT-185 induces apoptosis in primary MCL cells. We investigated whether KPT-185 could induce apoptosis in MCL cells from five primary samples (three with wild-type p53 and two with mutant p53, as determined by annexin V induction). The primary MCL cells were cultured in medium in the presence or absence of KPT-185 (100 or 400 nM) or 2.5 \text{\textmu}M Nutlin-3a. The p53 mutant samples were resistant to Nutlin-3a (Fig. 5), which was consistent with previously reported findings.\textsuperscript{26,27} The two p53-mutant MCL samples were less sensitive to 100 nM KPT-185 exposure than p53 wild-type MCL samples. However, when the concentration was increased to 1 \text{\textmu}M, the KPT-185 exposure induced apoptosis in 71% of the p53 mutant cells from patient 1. Of note, serum concentrations of >1 \text{\textmu}M KPT-330, a SINE XPO1 antagonist related to KPT-185 and currently in phase I clinical studies, are achievable at well-tolerated doses in humans.\textsuperscript{35,36} Data suggest that p53-mediated and transcription-dependent apoptosis is the primary signaling of SINE-induced apoptosis and that KPT-185 may induce p53-independent apoptosis at higher concentrations.

Absence of acquired TP53 mutations in KPT-adapted MCL cells. Nutlin-3a has been shown to induce or select TP53 mutations in p53 wild-type cancer cells, resulting in the development of subclones that are resistant to p53-mediated apoptosis.\textsuperscript{35,36} As KPT-185 appeared to activate p53-dependent apoptotic signaling in MCL cells, we investigated if long-term KPT-185 exposure induces or selects p53 mutant clones. Both Z-138 and JVM-2 cells were continuously exposed for 2 months to KPT-185 starting at ED\textsubscript{50}. The JVM-2 cells adapted to grow in the presence of KPT-185 in two independent experiments, as determined by more than 90% viability.
MDM2 inhibition may not sufficiently accumulate p53 into the nucleus and activate transcription-dependent p53 signaling in MCL. This is probably due to the E3 ubiquitin ligase activity of MDM2. MDM2 is the major ubiquitin ligase of p53, which promotes p53 proteasomal degradation both in the nucleus and the cytoplasm. Therefore, MDM2 inhibition may increase p53 levels in the cytoplasm, where p53 is unable to execute transcriptional activity. Although cytoplasmic p53 can mediate transcription-independent apoptosis in some circumstances, the quantitative contribution to p53-mediated apoptosis still remains unclear. In contrast, KPT-185 treatment activated transcription-dependent p53 signaling toward apoptosis in MCL cells. As increased XPO1 expression predicted poor survival in MCL patients, and MDM2 inhibition might not fully activate p53-mediated transcription, p53 activation by targeting XPO1 might offer a novel and plausible therapeutic strategy for MCL that retains wild-type p53. This hypothesis may be extended to solid cancers in which XPO1 overexpression is associated with poor prognosis. Interestingly, preliminary data in patients with advanced, relapsed/refractory MCL and other NHLs treated in the ongoing phase I study (clinicaltrials.gov NCT01607892) of the related SINE compound selinexor (KPT-330), show that XPO1 inhibition can induce responses even in heavily pretreated NHL patients. 

Fig. 4. Exportin-1 inhibitor KPT-185 induces apoptosis in primary mantle cell lymphoma cells. Primary cells from five patients were incubated for 12 h with KPT-185 or Nutlin-3a at their ED50 or 2 x ED50 concentrations, and transcripts were quantitated by real-time PCR. Data shown are from one of three independent experiments with similar results.

Fig. 5. Exportin-1 inhibitor KPT-185 induces apoptosis in primary mantle cell lymphoma cells. Primary cells from five patients were incubated for 72 h with Nutlin-3a (2.5 μM) or KPT-185 (100 or 1000 nM), and the annexin V-negative fractions were measured by flow cytometry. Three samples had wild-type p53 and two had mutant p53 (one had T155P and another Y205H).

(Trypan blue-negative cell percentage) at 8 weeks of exposure to x1.5 ED50. Acquired TP53 mutations were not detected in the SINE-adapted cells, suggesting that the mechanism of resistance is independent of TP53 mutations.

Discussion

We found that p53 is a determinant of apoptosis induction by the SINE XPO1 inhibitor KPT-185 in MCL. XPO1 carries ~230 XPO1 cargo proteins and many of them are responsible for MCL cell survival, proliferation, and cell death. Our data highlight the importance of p53 in the cellular survival network mediated by XPO1 cargos.

The nuclear export of p53 is cooperatively mediated by MDM2 and XPO1, and it is anticipated that their inhibition would lead to nuclear p53 retention and transcriptional activation of p53 target genes. However, our data suggest that...
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