BMK1 is involved in the regulation of p53 through disrupting the PML-MDM2 interaction

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Abstract
Promyelocytic leukemia protein (PML) modulates the p53 tumor suppressor through its interaction with p53 and MDM2. We found that activated BMK1 preferentially associates with PML isoform IV and disrupts PML-MDM2 interaction. Doxorubicin, a common chemotherapeutic agent, is known to promote PML-mediated p53 activation in part by promoting PML-dependent MDM2 nucleolar sequestration. We discovered that BMK1 deactivation coupled with doxorubicin synergistically enhanced MDM2 nucleolar sequestration and, consequently, promoted PML-mediated p53 up-regulation leading to tumor cell apoptosis in vitro and tumor regression in vivo. Collectively, these results not only suggest that BMK1 activity plays a role in suppressing p53 by blocking the interaction between PML and MDM2 but also implicate that pharmacological BMK1 inhibitor should significantly enhance the anti-cancer capacity of doxorubicin-based chemotherapy.

Keywords
BMK1; PML; p53

Introduction
In mammalian cells, the mitogen-activated protein (MAP) kinase signaling system consists of four distinct linear signaling cascades that terminate in activation of either ERK1/2, JNK, p38 or BMK1 (1-4). BMK1 shares 66% sequence homology with the kinase domain of ERK1/2. BMK1 and ERK1/2 both contain same TEY dual phosphorylation motif (5).
However, BMK1 has a unique activating loop structure and an uncommonly large C-terminal non-kinase domain (6). As the most recently discovered and least studied mammalian MAP kinase, BMK1 has so far been associated with general properties of human malignancies, including chemoresistance (7), uncontrolled proliferation (8), metastatic potential (9) and tumor-associated angiogenesis (10).

MDM2 is the major E3 ubiquitin ligase for p53 which facilitates p53 ubiquitination and consequent p53 degradation. As such, MDM2 is the key negative regulator of p53 (11). Substantial p53 up-regulation can be achieved by disrupting the MDM2-p53 interaction such as the phosphorylation of p53 and/or MDM2 as well as the sequestration of MDM2 or p53 to sub-nuclear compartments (11). Cellular stresses are known to utilize certain mechanism(s) described above to hinder the p53-MDM2 interaction thereby up-regulating p53. For example, the treatment of doxorubicin (a topoisomerase inhibitor), via PML, induces the nucleolar sequestration of MDM2 and subsequent p53 induction (12).

Our previous study demonstrated that upon activation BMK1 translocates from the cytosol into the nucleus, where it subsequently interacts with the promyelocytic leukemia protein (PML) in PML-nuclear bodies (NBs; multi-protein sub-nuclear structures) and acts to suppress tumor growth (13). PML (also known as MYL, RNF71, PP8675 or TRIM19) is a well-characterized anti-oncogenic protein, and has been described as a key regulator of a broad range of cellular functions, including inhibiting proliferation, inducing cellular senescence and apoptosis, and maintaining genomic stability (14). In addition, PML has been shown to dynamically associate with a multitude of different proteins within the PML-NB in response to specific stimuli such as that doxorubicin promotes PML interaction with MDM2 (12) and this PML-bound MDM2 is sequestered in the nucleolus, facilitating p53 induction. Nonetheless, the detailed mechanisms modulating the PML and MDM2 interaction and the following p53 induction remains unclear.

Results

BMK1 is involved in the regulation of p53 through PML

Previously, we described that short-term (2 hr) treatment with the BMK1 inhibitor XMD8-92 did not significantly increase the protein level or phosphorylation status of p53 in cells (13). However, a time course study (Figure 1a) revealed that long-term (32-40 hr) exposure to XMD8-92 led to up-regulation of cellular p53. Likewise, knock-down of cellular BMK1 by siRNA or shRNA caused up-regulation of p53 (Figure 1b and c).

Since many studies have reported that PML is an effective regulator of p53 (15-17) and BMK1 regulates PML activity (13), we hypothesized that BMK1 is capable of functionally modulating p53 through PML. Mouse (C54BL/6) embryonic fibroblast (MEF) cells lines with Pml+/+ and Pml−/− were used to determine whether p53 modulation by BMK1 occurred through PML. PML deficiency was associated with a significantly decreased p53 activity, which would have been otherwise induced by BMK1 inactivation (Figure 1d). In addition, when exogenous PML was introduced into Pml−/− MEF cells by recombinant PML adenovirus (Ad-PML), the BMK1 inhibition-induced up-regulation of p53 was restored.
(Figure 1e). These findings indicated that BMK1 might play a role in regulating p53 via PML.

**Among PML isoforms, BMK1 preferably but not exclusively associates with PML IV**

Considering that p53 can specifically interact with PML IV and has been reported to be regulated by PML (15-17), the ability of BMK1 to interact with various isoforms of PML (I – VI) (Figure 2a) was examined by immunoprecipitation (IP) using anti-BMK1 and anti-PML antibodies. IP results indicated that BMK1 could interact with all PML isoforms except PML V (Figure 2b). However, BMK1 exhibited the highest affinity for PML IV (Figure 2b). We extended our analysis of BMK1 and each of the PML isoforms by investigating the specific interactions in vivo using immunofluorescence (IF). As described in previous studies (13, 18), MEK5D (a constitutively active mutant of the kinase MEK5) phosphorylates BMK1 and can be used to maintain BMK1 activation; in contrast, MEK5A (a constitutively inactive mutant of MEK5) can inhibit the activation of BMK1 by diminishing its phosphorylation, similar to the specific BMK1 inhibitor activity of the XMD8-92 drug (Figure 2c). BMK1 activation was shown to correspond to its translocation from the cytoplasm to the nucleus (Figure 2d) (19, 20). IF data demonstrated that PML isoforms I-IV and VI colocalized with MEK5-activated BMK1 and that PML IV colocalization was that most robust. In agreement with the in vitro IP findings (Figure 2b), PML V did not colocalize with BMK1 at all (Figure 2d and e). These data raised the hypothesis that BMK1 might be involved in the regulation of p53 through affecting the interaction between p53 and PML.

**BMK1 may colocalize with p53 in PML-NBs and disrupt the interaction between PML and MDM2**

To verify the hypothesized mentioned above, co-IP and IF were applied to investigate the interactions between p53, BMK1 and PML. BMK1 or p53 were only detected in the IPs in which p53, BMK1 and PML were simultaneously present (Figure 3a). IF analysis of p53, PML and activated BMK1 indicated that the three proteins colocalized (Figure 3b and c). It appeared that BMK1 might indirectly associate with p53 via PML and BMK1 does not hamper the interaction between p53 and PML.

Furthermore, since many proteins that have been previously shown to dynamically associate with PML in PML-NB in response to specific stimuli (12, 15, 16, 21). We screened a collection of known PML-associated proteins using co-IP and IF assays to determine whether BMK1-mediated p53 regulation involved another PML-associated protein. The results indicated that the presence of activated BMK1 significantly disrupted the interaction between PML and MDM2 (Figure 3b-f). PML (or MDM2) could not be detected in the MDM2 (or PML) IP when PML, BMK1 and MDM2 were all present simultaneously (Figure 3d). Activated BMK1 abolished the co-localization of PML and MDM2 in cells (Figure 3e and f). Taking into account that the PML-DM2 interaction plays an important role in the activation of p53 (12, 22, 23), our data suggested that activated BMK1 might be involved in the regulation of p53 through disruption of the PML-DM2 interaction.

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There is a synergistic effect between doxorubicin and the BMK1 inhibitor

Doxorubicin, a topoisomerase inhibitor which is commonly used as a chemotherapeutic agent, is known to promote PML-mediated p53 activation by enhancing the PML-MDM2 interaction (12). As such, doxorubicin was applied to further investigate and verify the role of BMK1 in inhibiting p53 activation and the PML-MDM2 interaction. MEF, A549 (lung cancer) and HeLa (cervical tumor) cells were treated with doxorubicin, PD184352 (an ERK1/2 pathway inhibitor) and/or XMD8-92 (Figure 4a and b). Unlike PD184352, XMD8-92 showed substantial synergy with doxorubicin in up-regulating p53 (Figure 4a and b). In contrast, the synergistic effect was not observed in Pml−/− and Bmk1−/− MEFs. Bmk1−/− MEFs were also found to be more sensitive to doxorubicin than the Pml−/− MEFs (Figure 4c and d). As p53 induction is associated with programmed cell death, it was not surprising that the combinatorial doxorubicin and XMD8-92 treatment promoted significant apoptosis in tumor cells (Figure 4e). The synergistic anti-cancer effects elicited by the combination of BMK1 inhibitor and doxorubicin are most likely a result of BMK1 inhibitor alleviating activated BMK1-imposed suppression of doxorubicin-promoted PML tumor suppressor activity. Intriguingly, this combined treatment was able to overcome the human papillomavirus (HPV)-mediated p53 suppression by inducing p53 and apoptosis in HPV-positive HeLa cells (Figure 4b and e).

The BMK1 inhibitor enhances nucleolar sequestration of MDM2

Doxorubicin is known to promote PML-mediated MDM2 nucleolar sequestration (12). The observation of activated BMK1 interrupting the PML-MDM2 association suggested that blocking BMK1 activity should significantly enhance the PML-MDM2 interaction during doxorubicin treatment. Indeed, under low-dose doxorubicin treatment, XMD8-92 drastically increased the MDM2 nucleolar sequestration in Pml+/+ but not in Pml−/− MEF cells (Figure 5a-c). MDM2 sequestration in the nucleolus precludes its ability to facilitate ubiquitination (and targeted degradation) of p53; thus, the synergistic effect between doxorubicin and XMD8-92 to sequester MDM2 in the nucleolus was also associated with considerably decreased ubiquitination of p53 in cells (Figure 5d).

Pharmacological BMK1 inhibitor drastically enhances anti-cancer activity of doxorubicin

As described above, the BMK1 inhibitor XMD8-92 synergized with doxorubicin to induce p53 activity and apoptosis. Since doxorubicin is a well-known chemotherapeutic reagent currently in widespread clinical use, it was evaluated in animal tumor models to determine whether it could synergistically enhance the ability of the BMK1 inhibitor XMD8-92 to inhibit tumor development. We found that combinatorial treatment with XMD8-92 and doxorubicin produced synergistically increased p53 in tumors (Figure 6a). Importantly, this combinatorial treatment not only inhibited tumor growth but also caused significant regression in absolute tumor volume (Figure 6b). XMD8-92 alone has been shown to be insufficient for inducing apoptosis (13) in tumors; however, when XMD8-92 was applied in combination with doxorubicin the tumors exhibited considerably increased apoptotic events (Figure 6c). Taken together, these results demonstrated that XMD8-92 is capable of substantially promoting the anti-cancer effects of doxorubicin in animals bearing tumors.
Discussion

In this study, we found that activated BMK1 interrupts the MDM2 association with PML, leading to p53 stabilization. Conversely, deactivation of BMK1 promoted the PML-MDM2 interaction, resulting in p53 induction. Doxorubicin is known to promote MDM2 sequestration by PML, consequently up-regulating p53 (12). Combination treatment of tumor cells using BMK1 inhibitor and low-dose doxorubicin could significantly upregulate p53. These data indicate that activated BMK1 plays a critical role in blocking PML-dependent sequestration of MDM2, thereby inhibiting p53 induction, especially in cancer cells responding to doxorubicin treatment. In addition, as BMK1 is known to negatively modulate both PML and p53 tumor suppressor function and BMK1 is activated by a variety of commonly deregulated oncogenes (e.g. STAT3, Her2 and Ras), it is very likely that oncogene-induced BMK1 activation would increase the proliferation/survival/chemoresistance potentials of the tumor cells, especially those under doxorubicin treatment. This effect is likely to be due, at least in part, to BMK1 dampening of the anti-cancer capacity mediated by the PML-MDM2 interaction.

Most human cancers present with a deficient p53 signaling pathway. Approximately 40-50% of clinical cancer samples harbor inactivating mutations in p53. Moreover, almost half of all cancers retain the wild-type p53 allele, indicating that other mechanisms, such as inactivation of p53 by viral and deregulated host cellular products, contributed to the perturbation of this pathway. Herein, we demonstrated that a combination treatment of BMK1 inhibitor and doxorubicin overcame the wild-type p53 deactivation by E6 oncoprotein-induced p53 degradation (24) in HPV-positive cervical cancer cells (HeLa). This combination treatment not only synergistically induced p53 and apoptosis in these cancer cells, but also caused regression of this type of tumor in animals. Thus, it appears that this treatment might be useful in managing certain cancers carrying wild-type p53 that is being actively suppressed by factors such as HPV-E6 (24), MDM2 overexpression (25), or Arf deficiency (26) (which otherwise stabilizes p53).

Currently, doxorubicin (trade name Adriamycin; also known as hydroxydaunorubicin) is routinely applied to treat a wide range of cancers, including hematological malignancies, numerous types of carcinomas, and soft tissue sarcomas. The utility of this drug, however, elicits frequent cardiotoxic side effect and is life-threatening to cancer patients undergoing therapy (27). Our finding that augmenting low-dose doxorubicin with pharmacological BMK1 inhibitor can significantly enhance p53 induction and apoptosis suggests that this specific combinatorial treatment may not only substantially enhance the effectiveness of doxorubicin in treating cancer but also lower the dose requirement for doxorubicin, thereby reducing the adverse heart damage in cancer patients normally elicited by doxorubicin.

Materials and methods

Cell culture and transfection

HeLa, A549, 293FT, Pml+/- MEf, Pml−/− MEF, Bmk1+/+ MEF and Bmk1−/− MEF cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin and streptomycin at 37°C under a
humidified atmosphere of 5% CO₂. Transfections were performed using GenJet™ In Vitro DNA Transfection Reagent (Signagen Laboratories, Gaithersburg, MD) according to the manufacturer’s instruction.

**Immunoblotting and immunoprecipitation**

Immunoblotting and immunoprecipitation were carried out as previously described (13). Briefly, cells for immunoblotting were harvested in RIPA lysis buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF and 1mM sodium orthovanadate), or for immunoprecipitation in E1A lysis buffer (250 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% NP40, 5 mM EDTA, protease inhibitor cocktail (Roche, Indianapolis, IN), phosphatase inhibitor cocktail (Roche, Indianapolis, IN)). Immunoprecipitation was carried out by incubating lysates with the precipitating antibody for 6 hr and a subsequent 14 hr incubation with protein G beads (Invitrogen, Carlsbad, CA). Immune complexes were then washed three times in E1A lysis buffer and boiled in 2 × gel loading buffer. Proteins from total cell lysates or immunoprecipitates were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), then transferred to nitrocellulose membrane, blocked in 5% nonfat milk or bovine serum albumin, and blotted with the appropriate antibody.

**Immunofluorescence**

Briefly, cells were plated on coverslips pre-coated with type I collagen. After treatment, the cells were washed; all washes were carried out three times for 10 min each and with PBS, except where noted. Cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature. After washing, the cells were permeabilized using 0.2% Triton-X 100 in PBS for 10 min and blocked with 1% normal goat serum (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After washing, cells were incubated with rabbit antibody for 1.5 hr, and washing carried out with 1% normal goat serum in PBS. This was followed by 1.5 hr incubation with Alexa Fluor 488® or 568® goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). After washing with 1% normal goat serum in PBS, the cells were incubated for 1.5 hr with mouse antibody. A subsequent washing with 1% normal goat serum in PBS was followed by 1.5 hr incubation with Alexa Fluor 488® or 568® goat anti-mouse IgG (Invitrogen, Carlsbad, CA). A final quadruplicate washing was carried out with PBS alone before coverslips were mounted with a drop of mounting medium supplemented with DAPI (Vector Laboratories, Burlingame, CA) and sealed with clear nail polish.

**siRNA analysis**

Following the manufacturer’s protocol, siRNA analysis of BMK1 was performed using siGenome SMARTpool siRNA reagent (Dharmacon, Lafayette, CO) against BMK1 (5'-CAUGAACCUGCGCAGAUU-3', 5'-GCCCAGCGUGCUCGCAUCUCA-3', 5'-GAACUGUGACGCUAAGAUU-3' and 5'-AAACCAGUCUUUCGACAG-3'). Non-targeting siRNA (Dharmacon) was used as control.
Reagent and cell lines

Anti-BMK1 antibody used for immunoblotting and immunoprecipitation was described previously (28). The anti-BMK1 rabbit monoclonal used for immunofluorescence, and the anti-phospho-BMK1 (T218/Y220), anti-ERK1/2, anti-phospho ERK1/2 (T202/Y204), anti-Ubiquitin, anti-p21 and anti-p53 antibodies were from Cell Signaling (Beverly, MA). Anti-human PML, anti-HA and anti-MDM2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Nucleolin antibody was from Abcam (Cambridge, MA). Anti-mouse PML and anti-GAPDH antibodies were, respectively, from Upstate (Millipore, Billerica, MA) and Calbiochem (San Diego, CA). p3113 GST-p53 (29), pCMV-Neo-Bam p53 (30), pGEX-4T MDM2 WT (31) and pCMV-myc3-HDM2 (32) were from Addgene (Cambridge, MA). Ad-EV, Ad-PML, pCDNA3 FLAG-tagged BMK1, HA-tagged MEK5D and MEK5A-expressing vectors were described previously (13, 18, 33). FLAG-tagged PML isoforms (34) were a kind gift from Dr. Leppard at the University of Warwick (Coventry, United Kingdom). Expression vectors for GST-BMK1 and PML isoforms were produced by cloning the relative sequences into pGEX (GE Healthcare, Piscataway, NJ). PML null and control MEF lines were generous gifts from Dr. Myung Kim at the National Institutes of Health (NIH; Bethesda, MD) and Dr. Giovanni Blandino at the Regina Elena Cancer Institute (Rome, Italy). BMK1 null and control MEF lines were raised as described in our previous study (35). BMK1 control and knockdown A549 cell lines were established using empty vector--pGIPZ and pGIPZ-shRNAmir BMK1 (Openbiosystems, Huntsville, AL), respectively. PD184352 and doxorubicin were from Fisher Scientific (Fisher Scientific, Pittsburgh, PA). Recombinant adenoviral particles were generated as described previously (36).

Isolation of nucleoli

Nucleoli were prepared from Pml+/+ MEF and Pml−/− MEF cells using a slightly modified protocol from previous study (37). Specifically, protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Roche, Indianapolis, IN) were added to all of the buffers described (37).

TUNEL assay

The in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) was used for TUNEL assay, following the manufacturer’s instructions.

HeLa and A549 xenograft model

The following animal handling and procedures were approved by the Scripps Institutional Animal Care and Use Committee and followed NIH guidelines. Cultured cells (1 × 10^6 HeLa cells or 2 × 10^6 A549 cells) were resuspended in DMEM and injected subcutaneously into the right flank of six week-old Nod/Scid mice. After 10 days, these tumor-bearing mice were randomized into four groups (n = 9 per group): Control, XMD8-92, Doxorubicin and Doxorubicin+XMD8-92. Mice were injected every two days with either vehicle, doxorubicin (1 mg/kg) and/or XMD8-92 (50 mg/kg). In each group, three mice were used for western blot and TUNEL assays; six mice for tumor volume measurement. The control group mice were sacrificed on day 15 (HeLa) or day 21 (A549). Tumor size was measured.
by caliper and tumor volume was calculated by using the formula: \(0.52 \times L \times W^2\), where \(L\) was the longest diameter and \(W\) was the shortest diameter.

**Statistical analysis**

\(p\) values were calculated using the Student’s \(t\)-test.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**

1. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001; 410:37–40. [PubMed: 11242034]
2. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 2002; 298:1911–2. [PubMed: 12471242]
3. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001; 22:153–83. [PubMed: 11294822]
4. Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. Oncogene. 2007; 26:3100–12. [PubMed: 17496909]
5. Zhou G, Bao ZQ, Dixon JE. Components of a new human protein kinase signal transduction pathway. The Journal of biological chemistry. 1995; 270:12665–9. [PubMed: 7759517]
6. Lee JD, Ulevitch RJ, Han J. Primary structure of BMK1: a new mammalian map kinase. Biochemical and biophysical research communications. 1995; 213:715–24. [PubMed: 7646528]
7. Weldon CB, Scandurro AB, Rolfe KW, Clayton JL, Elliott S, Butler NN, et al. Identification of mitogen-activated protein kinase kinase as a chemoresistant pathway in MCF-7 cells by using gene expression microarray. Surgery. 2002; 132:293–301. [PubMed: 12219026]
8. Esperis-Ogando A, Diaz-Rodriguez E, Montero JC, Yuste L, Crespo P, Pandiella A. Erk5 participates in neuregulin signal transduction and is constitutively active in breast cancer cells overexpressing ErbB2. Mol Cell Biol. 2002; 22:270–85. [PubMed: 11739740]
9. Mehta PB, Jenkins BL, McCarthy L, Thilak L, Robson CN, Neal DE, et al. MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion. Oncogene. 2003; 22:1381–9. [PubMed: 12618764]
10. Hayashi M, Fears C, Eliceiri B, Yang Y, Lee JD. Big mitogen-activated protein kinase 1/ extracellular signal-regulated kinase 5 signaling pathway is essential for tumor-associated angiogenesis. Cancer Res. 2005; 65:7699–706. [PubMed: 16140937]
11. Oren M. Regulation of the p53 tumor suppressor protein. J Biol Chem. 1999; 274:36031–4. [PubMed: 10593882]
12. Bernardi R, Scaglioni PP, Bergmann S, Horn HF, Vouwen KH, Pandolfi PP. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat Cell Biol. 2004; 6:665–72. [PubMed: 15195100]
13. Yang Q, Deng X, Lu B, Cameron M, Fears C, Patricelli MP, et al. Pharmacological inhibition of BMK1 suppresses tumor growth through promyelocytic leukemia protein. Cancer cell. 2010; 18:258–67. [PubMed: 20832753]
14. Bernardi R, Pandolfi PP. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nat Rev Mol Cell Biol. 2007; 8:1006–16. [PubMed: 17928811]

15. Pearson M, Carbone R, Sebastiani C, Cioce M, Fagioli M, Saito S, et al. PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature. 2000; 406:207–10. [PubMed: 10910364]

16. Guo A, Salomoni P, Luo J, Shih A, Zhong S, Gu W, et al. The function of PML in p53-dependent apoptosis. Nat Cell Biol. 2000; 2:730–6. [PubMed: 11025664]

17. Louria-Hayon I, Grossman T, Sionov RV, Alsheich O, Pandolfi PP, Haupt Y. The promyelocytic leukemia protein protects p53 from Mdm2-mediated inhibition and degradation. J Biol Chem. 2003; 278:33134–41. [PubMed: 12810724]

18. Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. The EMBO journal. 1997; 16:7054–66. [PubMed: 9384584]

19. Yan C, Luo H, Lee JD, Abe J, Berk BC. Molecular cloning of mouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains. The Journal of biological chemistry. 2001; 276:10870–8. [PubMed: 11139578]

20. Kondoh K, Terasawa K, Morimoto H, Nishida E. Regulation of nuclear translocation of extracellular signal-regulated kinase 5 by active nuclear import and export mechanisms. Mol Cell Biol. 2006; 26:1679–90. [PubMed: 16478989]

21. Ferbeyre G, de Stanchina E, Querido E, Baptiste N, Prives C, Lowe SW. PML is induced by oncogenic ras and promotes premature senescence. Genes Dev. 2000; 14:2015–27. [PubMed: 10950866]

22. Zhu H, Wu L, Maki CG. MDM2 and promyelocytic leukemia antagonize each other through their direct interaction with p53. J Biol Chem. 2003; 278:49286–92. [PubMed: 14507915]

23. Kurki S, Latonen L, Laiho M. Cellular stress and DNA damage invoke temporally distinct Mdm2, p53 and PML complexes and damage-specific nuclear relocalization. J Cell Sci. 2003; 116:3917–25. [PubMed: 12915590]

24. Scheffner M, Werness BA, Huibregts JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990; 63:1129–36. [PubMed: 2175676]

25. Polsky D, Bastian BC, Hazan C, Melzer K, Pack J, Houghton A, et al. HDM2 protein overexpression, but not gene amplification, is related to tumorigenesis of cutaneous melanoma. Cancer Res. 2001; 61:7642–6. [PubMed: 11606406]

26. Haluska FG, Hodi FS. Molecular genetics of familial cutaneous melanoma. J Clin Oncol. 1998; 16:670–82. [PubMed: 9469357]

27. Bisht S, Maitra A. Dextran-doxorubicin/chitosan nanoparticles for solid tumor therapy. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2009; 1:415–25. [PubMed: 20049807]

28. Abe J, Kusuhara M, Ulevitch RJ, Berk BC, Lee JD. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. J Biol Chem. 1996; 271:16586–90. [PubMed: 8663194]

29. Huibregts JM, Scheffner M, Howley PM. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. Embo J. 1991; 10:4129–35. [PubMed: 1661671]

30. Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science. 1990; 249:912–5. [PubMed: 2144057]

31. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol. 2001; 3:973–82. [PubMed: 11715018]

32. Zhang Y, Wolf GW, Bhat K, Jin A, Allio T, Burkhart WA, et al. Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Mol Cell Biol. 2003; 23:8902–12. [PubMed: 14612427]

33. Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ, Lee JD. Bmkl/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature. 1998; 395:713–6. [PubMed: 9790194]

34. Beech SJ, Lethbridge KJ, Killick N, McGlincy N, Leppard KN. Isoforms of the promyelocytic leukemia protein differ in their effects on ND10 organization. Exp Cell Res. 2005; 307:109–17. [PubMed: 15922731]

Oncogene. Author manuscript; available in PMC 2013 December 27.
35. Hayashi M, Kim SW, Imanaka-Yoshida K, Yoshida T, Abel ED, Eliceiri B, et al. Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. The Journal of clinical investigation. 2004; 113:1138–48. [PubMed: 15085193]

36. Hayashi M, Tapping RI, Chao TH, Lo JF, King CC, Yang Y, et al. BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of serum and glucocorticoid-inducible kinase. J Biol Chem. 2001; 276:8631–4. [PubMed: 11254654]

37. Andersen JS, Lyon CE, Fox AH, Leung AK, Lam YW, Steen H, et al. Directed proteomic analysis of the human nucleolus. Current biology: CB. 2002; 12:1–11. [PubMed: 11790298]
Figure 1.
BMK1 is involved in the regulation of p53 through PML. (a) A549 cells were treated with XMD8-92 (4 μM) for 0, 8, 16, 24, 32 and 40 hours. Cell lysates were analyzed by western blot using anti-p53 or anti-GAPDH antibodies. (b) A549 cells were transfected with control or BMK1-specific siRNA for 48 hr. Cell lysates were analyzed by western blot using anti-BMK1, anti-p53 or anti-GAPDH antibodies. (c) BMK1 knockdown A549 cell line was established using pGIPZ-shRNA mir BMK1. Control A549 cell line was generated using empty vector pGIPZ. Cell lysates were analyzed by western blot using anti-BMK1, anti-p53 or anti-GAPDH antibodies. (d) Pml<sup>+/+</sup> MEF and Pml<sup>−/−</sup> MEF cells were treated with or without XMD8-92 (4 μM) for 36 hr. Cell lysates were analyzed by western blot using anti-BMK1, anti-PML, anti-p53 or anti-GAPDH antibodies. (e) Pml<sup>−/−</sup> MEF cells were infected with either empty adenovirus (Ad-EV) or recombinant adenovirus encoding PML (Ad-PML). Three days later, cells were treated with or without XMD8-92 for 36 hr. Cell lysates were analyzed by western blot using anti-BMK1, anti-PML, anti-p53 or anti-GAPDH antibodies.
Figure 2.
BMK1 preferentially, but not exclusively, associates with PML IV. (a) Primary structure of PML I – VI. (b) Recombinant proteins BMK1 and PML isoforms were incubated and immunoprecipitated. The resultant BMK1 and PML isoforms immunoprecipitates were analyzed by western blot using anti-PML or anti-BMK1 antibodies. (c) 293FT cells were transfected with MEK5D (HA-tagged) or MEK5A (HA-tagged) and treated with or without XMD8-92 (4 μM). The resultant lysates were analyzed by western blot using anti-BMK1, anti-phospho BMK1 (T218/Y220), anti-HA and anti-GAPDH antibodies. (d) Fluorescent
microscopy images of HeLa cells transfected with expression vectors encoding BMK1, PML isoforms, MEK5A or MEK5D. These cells were stained with anti-BMK1 (green, first-column panels) or anti-PML (PG-M3) antibodies (red, second-column panels). Nuclei staining by DAPI (blue, third-column panels). Merged images (yellow, fourth-column panels). Scale bar = 10 μm. (e) Enlarged fluorescent microscopy images, corresponding to numbers 1-4 in (d).
Figure 3.
BMK1 colocalizes with p53 in PML-NBs and interrupts the interaction between PML and MDM2. (a) Recombinant proteins BMK1, p53 and PML IV were incubated and immunoprecipitated. The resultant immunoprecipitates were analyzed by western blot using anti-PML, anti-p53 or anti-BMK1 antibodies. (b) Fluorescent microscopy images of HeLa cells transfected with expression vectors encoding BMK1, PML, p53, MEK5A or MEK5D. These cells were stained with anti-BMK1 (first-column panels), anti-p53 (second-column panels) or anti-PML (third-column panels) antibodies. Nuclei were staining by DAPI (fourth-column panels). Merged images (fifth-column panels). Scale bar = 10 μm. (c) Enlarged fluorescent microscopy images, corresponding to numbers 1-4 in (b). White arrows indicate BMK1 shows the interaction p53 through PML. (d) Recombinant BMK1, MDM2 and PML proteins were incubated and immunoprecipitated. The resultant immunoprecipitates were analyzed by western blot using anti-PML, anti-MDM2 or anti-BMK1 antibodies. (e) Fluorescent microscopy images of HeLa cells transfected with expression vectors encoding BMK1, PML, MDM2, MEK5A or MEK5D. These cells were stained with anti-BMK1 (first-column panels), anti-MDM2 (second-column panels) or anti-
PML (third-column panels) antibodies. Nuclei staining by DAPI (fourth-column panels). Merged images (fifth-column panels). Scale bar = 10 μm. (f) Enlarged fluorescent microscopy images, corresponding to numbers 5-8 in (e). White arrows indicate BMK1 interrupts the interaction between PML and MDM2.
Figure 4.
There is a synergistic effect between doxorubicin and the BMK1 inhibitor. (a) MEF cells were treated with or without XMD8-92 (4 μM), PD184352 (2 μM) or doxorubicin (600 nM) for 18 hr. Cell lysates were analyzed by western blot using anti-BMK1, anti-ERK1/2, anti-phospho ERK1/2 (T202/Y204), anti-p53 or anti-GAPDH antibodies. The amount of the p53 protein in MEF cells without treatment, normalized against GAPDH, was considered as 1.0. n = 5; ± SEM; **p < 0.01. (b) A549 and HeLa cells were treated with or without XMD8-92 (4 μM), PD184352 (2 μM) or doxorubicin (600 nM) for 18 hr. Cell lysates were analyzed by western blot using anti-BMK1, anti-phospho-BMK1 (T218/Y220), anti-ERK1/2, anti-phospho ERK1/2 (T202/Y204), anti-p53 or anti-GAPDH antibodies. The amount of the p53 protein in cells without treatment, normalized against GAPDH, was considered as 1.0. n = 5; ± SEM; **p < 0.01. (c) Pml+/+ MEF and Pml−/− MEF cells were treated with or without XMD8-92 (4 μM) or doxorubicin (600 nM) for 18 hr. Cell lysates were analyzed by western blot using anti-BMK1, anti-p53 or anti-GAPDH antibodies. The amount of the p53 protein in cells without treatment, normalized against GAPDH, was considered as 1.0. n = 5; ± SEM; **p < 0.01. (d) Bmk1+/+ MEF and Bmk1−/− MEF cells were treated with or without XMD8-92 (4 μM) or doxorubicin (600 nM) for 18 hr. Cell lysates were analyzed by western
blot using anti-BMK1, anti-p53 or anti-GAPDH antibodies. The amount of the p53 protein in cells without treatment, normalized against GAPDH, was considered as 1.0. n = 5; ± SEM; **p < 0.01. (e) Fluorescent microscopy images of HeLa and A549 cells treated with vehicle (control), XMD8-92, doxorubicin or doxorubicin+XMD8-92 followed by TUNEL assay. n = 5 slice imaged and one representative image shown.
Figure 5.
The synergistic effect between doxorubicin and the BMK1 inhibitor enhances nucleolar sequestration of MDM2. (a) *Pml*<sup>+/−</sup> MEF and *Pml*<sup>−−</sup> MEF cells were treated with XMD8-92 (4 μM) and/or doxorubicin (300 nM) for about 24 hr and stained using anti-PML, anti-MDM2, and anti-Nucleolin antibodies, as noted. Arrows indicate cells for which images are enlarged and shown in (b). Scale bar = 10 μm. (b) Enlarged fluorescent microscopy images of specific cells in (a). Arrows indicate translocation of MDM2 to the nucleoli. (c) Nucleoplasm and nucleoli from *Pml*<sup>+/−</sup> MEF and *Pml*<sup>−−</sup> MEF cells, which were treated with XMD8-92 (4 μM) and/or doxorubicin (300 nM) for about 24 hr, were isolated as described in materials and methods. Western blot with anti-p53, anti-MDM2 anti-PML, anti-BMK1 or anti-Nucleolin antibodies was used to analyze extracts of nucleoplasm and nucleoli from *Pml*<sup>+/−</sup> MEF and *Pml*<sup>−−</sup> MEF. (d) Endogenous p53 was immunoprecipitated from cell lysates of WT MEF cells, which were treated with XMD8-92 (4 μM) and/or doxorubicin (300 nM) for about 20 hr. The ubiquitination of p53 in these immunoprecipitates was analyzed using anti-ubiquitin antibody.
Figure 6.
Pharmacological BMK1 inhibitor significantly enhances anti-cancer activity of doxorubicin. (a) Tumor homogenates from Nod/Scid mice subcutaneously injected with HeLa or A549 cells and treated with control, doxorubicin, XMD8-92 or doxorubicin+XMD8-92 for 30 hr were used in western blot to detect BMK1, p53 and GAPDH expression. n = 3 mice per group. (b) Mouse HeLa and A549 xenograft models were established as described in materials and methods and were randomized into 4 groups (Control, Doxorubicin, XMD8-92 and Doxorubicin+XMD8-92). These Mice were injected with vehicle, doxorubicin (1 mg/kg) and/or XMD8-92 (50 mg/kg) as indicated every two days. The HeLa and A549 control group mice were sacrificed on day 15 and 21 respectively. Tumor size was measured and tumor volume was calculated every two days. n = 6 mice; ± SEM; *\(p < 0.05\), **\(p < 0.01\). (c) Fluorescent microscopy images of HeLa or A549 tumor sections (analyzed by TUNEL assay) from control, XMD8-92, doxorubicin and doxorubicin+XMD8-92 treated mice. n = 3 mice per group, n = 5 slices each. Scale bar = 10 μm.