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14. ABSTRACT

Cyclin-dependent kinase inhibitor VMY-1-103 induces a G2/M cell cycle arrest and apoptosis in prostate cancer cell lines. Cancer cell lines, including prostate cancer, show a differential sensitivity to VMY-1-103 that correlates with p53 status. In addition, VMY-1-103 sensitivity increases in cancer cell lines as compared with normal cell lines, regardless of p53 status. Knockdown experiments in LNCaP cells show a reduced sensitivity to VMY-1-103 by resulting in a decreased cell death and this result can be rescued by the addition of wild-type p53. Transient transfections of wild-type p53 into p53-null PC-3 cells resulted in increased cell death upon VMY treatment. Furthermore, PRIMA-1 pre-treatment restored p53 activity in p53-mutant DU145 cells and sensitized them to VMY-mediated cell death. As compared with other solid tumors, only a small percentage of prostate cancer cases contain p53 mutations. Therapeutically, this is important as a majority of prostate cancer patients could benefit from VMY.

15. SUBJECT TERMS
P53, apoptosis, cyclin-dependent kinase inhibitor

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Introduction:

The mammalian cell cycle is a tightly regulated process that controls cell division and replication. The tumor suppressor p53 plays a crucial role in both the G1/S and mitotic checkpoints, as well as in apoptosis in response to DNA damage. P53 activates p21, which is an inhibitor of cdk/cyclin E complexes, to cause cell cycle arrest. P53 can also function as a mediator of apoptosis by activating either the intrinsic or extrinsic pathway of apoptosis via transcriptional targets such as Bax, Bid, and TRAIL R4/5 (1). Deregulation of the cell cycle has been described in many human tumors, including prostate cancer (2). In prostate cancer specifically, cyclinD1 has been shown to be upregulated while down-regulation is commonly seen in cyclin-dependent kinase (cdk) inhibitors such as $p16^{INK4A}$ and $p27^{Kip1}$ (3). Therefore, small molecule cyclin dependent kinase (cdk) inhibitors may have the ability to block tumor progression in prostate cancer. We have developed a novel analog of purvalanol B termed VMY-1-103 (VMY) that functions as a potent cdk1 and cdk2 inhibitor. VMY shows enormous potential as a therapeutic agent for prostate cancer treatment.

Body:

Aim 1 is to test VMY-1-103 on a panel of human prostate cancer cell lines and correlate activity with p53 status. VMY-1-103 was tested on a large panel of cancer and normal cell lines. These cell lines include prostate cancer cell line LNCaP, which is p53 wild-type, PC3 cells, which are null, and DU145 cells that contain p53 mutations. VMY-1-103 sufficiently caused an increase in cells in G2/M and greater than 40% cell death in LNCaP cells ($p<0.05$) as measured by trypan blue exclusion in 18 hours. In contrast the parent compound, purvalanol B, was only able to cause 2% cell death in these cells. Furthermore, less than 5% cell death was seen upon treatment of the PC3 and DU145 cells, although a G2/M arrest was seen in both cell types. Cell death in LNCaP cells was confirmed as apoptosis by an apoptosis protein expression array. Results showed that VMY treatment led to a significant increase in PARP cleavage and cleaved caspase 3, which are clear markers of apoptosis. In addition, VMY treatment led to an increase in phosphorylated levels of p53 (S15, S46, and S392) as well as total p21 levels, suggesting p53 is important in the apoptotic response following VMY treatment (4). Interestingly, VMY is able to induce a cell cycle arrest at the G2/M checkpoint in all cells, but the apoptotic factor seems to be related to p53 status.

Additional wild-type p53 cancer cell lines, PC12, A172, MCF7, and COLO-357, were all sensitive to VMY-1-103 treatment at similar rates as LNCaPs. In addition, other p53 mutant cell lines ASPC1, MIA-PaCa1, MDA231, and T98G, had less than 5% cell death following treatment. Furthermore, four additional prostate cell lines were generated from two patients; two normal cell lines (029N and 003N) and two tumor lines (030T and 004T). These cells were generated using methods developed at Georgetown University and all of these cell lines are p53 wild-type (5). Interestingly, the tumor cell line is more sensitive to cell death with VMY treatment than the normal cells (figure 1). Also, p21 was only expressed in the normal cell lines, and p21 levels increased upon VMY treatment, suggesting cell cycle arrest was occurring. To investigate this, cell cycle analysis was performed on all these cell lines following VMY treatment and all cell lines, both normal and tumor, arrested in G2/M upon VMY treatment (figure 2).

Our research has shown that VMY can cause chromosomal abnormalities and induce mitotic catastrophe in DAOY medulloblastoma cells, which have a p53 mutation (6). In order to examine if this holds true in prostate cancer cell lines, I stably transfected LNCaP and DU145 cells with a GFP HistoneH2B construct. I then performed time-course live cell imaging as previously described (6). In contrast to what was observed with the DAOY cells, no mitotic castrophe or delay of mitosis was seen. In addition, I performed confocal microscopy on LNCaP and DU145 cells stained with Aurora A and did not observe any mitotic abnormalities. Due to these results, I did not continue exploring the effects of VMY on these cells and did not do live imaging of the p53 knockdown or overexpressed cells as proposed as Aim 2d.

Aim 2 is to determine if wild-type p53 is required for VMY-1-103-induced apoptosis. To test this, I first performed siRNA knockdown of p53 in wild-type LNCaP cells (figure 3a). Briefly, cells were incubated with either adenovirus alone or adenovirus containing p53 siRNA. I next treated these knockdown cells with VMY and found that reduction in wild type levels of p53 greatly reduced the ability of VMY to cause cell death in these cells (figure 3b). In addition, I performed an apoptosis protein expression array on the p53 knockdown cells with and without VMY treatment and found that apoptosis was greatly reduced in the knockdown cells following VMY treatment as measured by Bax, cleaved Caspase 3, and TRAIL DR5 (figure 3d). Furthermore, I performed an siRNA rescue experiment in which I knocked down p53, transfected with a WT expression vector, and then treated cells with VMY. Ability to cause cell death was rescued as seen by an increase in cell death (figure 3c).

I next overexpressed WT p53 in PC3 (null) cells, which greatly sensitized them to VMY-induced cell death. I also transiently transfected mutant p53 (245), which did not affect cell death upon VMY treatment as compared with GFP transfection alone (figure 4). Because DU145 cells inherently have two p53 mutations with unknown functions, I could not simply overexpress a wild-type vector in these cells. Instead, I pre-treated these cells with a drug called PRIMA-1, which can restore wild-type function of p53 in cells with mutations in the DNA binding domain, such as DU145 (7). Upon treatment with PRIMA-1, p21 levels are restored in DU145, suggesting restoration of p53 function (figure 5a). Following PRIMA-1 pretreatment, I treated cells with flavopiridol, purvalanol B, or VMY and found that there was an increased sensitivity to cell death (figure 5b), although no changes in the cell cycle distribution as measured by flow cytometry. Furthermore, PRIMA-1 pre-treatment led to an increase in PARP cleavage upon VMY treatment, confirming an increase in apoptosis upon restoration of WT p53 (figure 5c).

Because I determined that WT p53 plays an important role in causing cell death upon VMY treatment in these cells, I next tested if mutant p53 constructs had the same effect. I created stable tet-on inducible LNCaP cells overexpressing mutant p53 at hotspot mutation sites 245 and 175 (Figure 6a). Upon VMY treatment, the overexpression of a mutant p53 construct did not change the cell cycle profile or ability of VMY to cause cell death. Because wild-type p53 is still present in these cells, I next knocked down wt p53 using siRNA, and then induced the mutant constructs using tetracycline. The knockdown of p53 alone inhibited the effect of VMY on halting the cell cycle and this effect was not rescued by the induction of either mutant construct (Figure 6b).

The original Aim 3 was to test VMY-1-103 in prostate cancer xenograph mouse models. Because we were able to use a novel method to establish primary prostate normal and tumor cells from patients, we believed this was stronger evidence for VMY's activity than animal experiments would be. In addition, new data emerged that gave great insight into the mechanism by which VMY was active as autophagy was found to play a large role in the activity of VMY in prostate cells. Autophagy is emerging as an important mediator of cell death in cancer cells and has been shown to be regulated by p53 (9). The function of p53 in connection to autophagy is not fully clear, as p53 can inhibit autophagy in normal cells (10) but also activate autophagy during apoptosis in cells that have been exposed to genotoxic stress, such as many chemotherapeutic agents (11). Because VMY causes apoptosis in p53 wildtype cells, the caspase requirement was tested in these cells to confirm if the intrinsic or extrinsic pathway of apoptosis was occurring. Interestingly, chemical inhibition of both caspases 8 and 9 does not fully reverse cell death following VMY treatment (figure 7), signifying that an additional mechanism of cell death besides apoptosis was occurring.

The fluorescent compound, Acridine Orange, is commonly used as a readout of autophagy. When LNCaP cells were exposed to it in the presence of VMY for 18 hours, confirmation of autophagasomes in the cytoplasm were found. LC3-I, which is normally located in the cellular microtubules, relocates to the autophagosomal membranes when autophagy is activated. When LNCaP cells were transfected with an LC3-GFP construct and treated with VMY, this re-localization was seen by fluorescent microscopy (figure 8), confirming that VMY was causing autophagy in p53 wildtype cells. When LNCaP cells were pre-treated with a p53 siRNA construct, autophagy was not activated following VMY treatment (figure 9). This suggests that the autophagic response that VMY is causing in prostate cancer cells is p53-dependent. A manuscript is currently being finalized including all of the p53 and autophagy findings from Aims 2 and 3 in prostate cancer cells.

Key Research Accomplishments:

- Expanded testing of VMY-1-103 to many cancer and normal cell lines.
- Strengthened correlation between efficacy of VMY-1-103 to cause apoptosis and p53 status in cell lines.
- Showed that knockdown of p53 in wild-type LNCaP cells greatly reduces sensitivity to VMY. This effect can also be rescued by addition of a wild-type p53 vector following siRNA knockdown. Mutant p53 constructs did not rescue this effect.
- Further confirmed that wild type p53 is required for VMY-mediated apoptosis by restoring wild type p53 function in p53 mutant DU145 cells, which greatly increased their sensitivity to the drug. Furthermore, transient transfection of wild-type p53 in p53 null PC3 cells rendered them sensitive to VMY.
- Expanded testing of VMY-1-103's cell cycle effect to primary human cancer and normal cell lines.
- Showed that the cell death response of VMY was not completely caspase dependent.

- Confirmed that VMY was causing autophagy in prostate cancer cells and that this process was also p53-dependent.

Reportable Outcomes:

- First author publication:
  - **Ringer L**, Sirajuddin P, Heckler M, Ghosh A, Suprynowicz F, Yenugonda VM, Brown ML, Toretsky JA, Uren A, Lee Y, Macdonald TJ, Rodriguez O, Glazer RI, Schlegel R, Albanese C. VMY-1-103 is a novel CDK inhibitor that disrupts chromosome organization and delays metaphase progression in medulloblastoma cells. **Cancer Biol Ther**. 2011 Nov 1;12(9):818-26
- Other publications:
  - Beauchamp EM, **Ringer L**, Bulut G, Sajwan KP, Hall MD, Lee YC, Peaceman D, Ozdemirli M, Rodriguez O, Macdonald TJ, Albanese C, Toretsky JA, Uren A. Arsenic trioxide inhibits human cancer cell growth and tumor development in mice by blocking Hedgehog/GLI pathway. **J Clin Invest**. 2011 Jan 4;121(1):148-60
  - Sirajuddin P, Das S, **Ringer L,** Rodriguez OC, Sivakumar A, Lee YC, Uren A, Fricke ST, Rood B, Ozcan A, Wang SS, Karam S, Yenugonda V, Salinas P, Petricoin E 3rd, Pishvaian M, Lisanti MP, Wang Y, Schlegel R, Moasser B, Albanese C. Quantifying the CDK inhibitor VMY-1-103's activity and tissue levels in an in vivo tumor model by LC-MS/MS and by MRI. Cell Cycle. 2012 Sept 14;11(20).
- Poster Presentations:
  - NIH National Graduate Student Research Conference. CDK inhibitor VMY-1-103 causes p53-dependent apoptosis in prostate cancer cells. October 9th, 2012.
  - American Association for Cancer Research Annual Meeting, Chicago IL. Role of p53 in CDK Inhibitor VMY-1-103-mediated Apoptosis . March 31st – April 4th, 2012.
  - Georgetown University Student Research Day. Role of p53 in CDK Inhibitor VMY-1-103-mediated Apoptosis. March 1st, 2012 Washington, DC.
  - Georgetown University Student Research Day. CDK Inhibitor VMY-1-103 Causes Cell Cycle Arrest and Apoptosis in Cancer Cell Lines. April 7th, 2011 Washington, DC.
  - Cell cycle Regulators/Inhibitors & Cancer. CDK Inhibitor VMY-1-103 Causes Cell Cycle Arrest and Apoptosis in Cancer Cell Lines. February 5th-8th, 2011 Vienna, Austria.

Conclusion:

Cyclin-dependent kinase inhibitor VMY-1-103 induces a G2/M cell cycle arrest and apoptosis in prostate cancer cell lines. Cancer cell lines, including prostate cancer, show a differential sensitivity to VMY-1-103 that correlates with p53 status. In addition, VMY-1-103 sensitivity increases in cancer cell lines as compared with normal cell lines, regardless of p53 status. This has important therapeutic implications for the treatment of prostate cancer patients. Knockdown experiments in LNCaP cells show a reduced sensitivity to VMY-1-103 by resulting in a decreased cell death and this result can be rescued by the addition of wild-type p53. Transient transfections of wild-type p53 into p53-null PC-3 cells resulted in increased cell death upon VMY treatment. Furthermore, PRIMA-1 pre-treatment restored p53 activity in p53-mutant DU145 cells and sensitized them to VMY-mediated cell death. In addition, this cell death response is also at least partially caused by p53-dependent autophagy As compared with other solid tumors, only a small percentage of prostate cancer cases contain p53 mutations (8). Therapeutically, this is important as a majority of prostate cancer patients could benefit from VMY treatment. Furthermore, p53 mutations are generally only seen in prostate cancer patients who have very aggressive tumors. Therefore, VMY may be a more useful therapeutic to use to treat earlier stages of prostate cancer than aggressive, metastatic cancer. Further studies are ongoing to test VMY in aggressive tumors, either by modulating the p53 status in the tumors or by combining VMY with other therapeutic agents.

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Supporting Data:

Figure 1: VMY-1-103 causes a larger amount of cell death in primary tumor cells compared with normal adjacent cells.

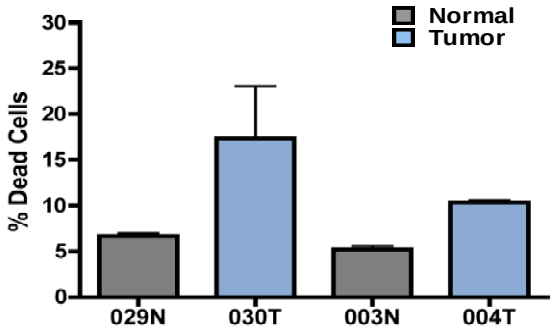

Figure 1: Percentage of cell death following 18hr of VMY treatment as quantified by trypan blue dye exclusion. 029N (normal) and 030T (tumor) are one patient and 003N (normal) and 004T (tumor) are from another patient. Cell death significantly increases in tumor cells as compared with the matched sets of normal cells.

Figure 2: VMY-1-103 cell cycle arrest in normal and tumor prostate cells but only induces p21 in the normal cell lines.

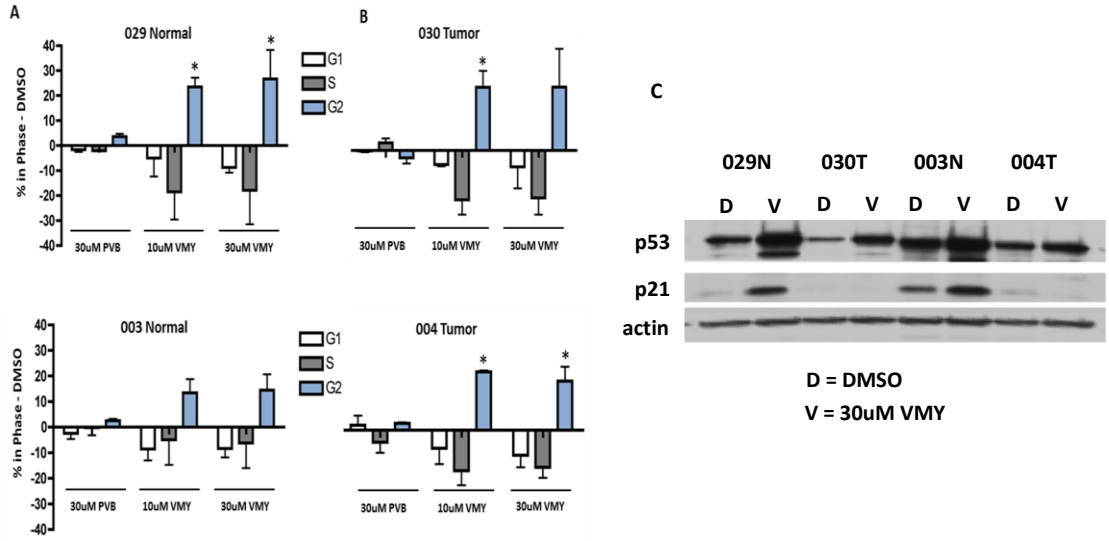

Figure 2: A. 029 and 003 normal prostate cell lines were treated with VMY for 18 hours. Following treatment, cells were fixed and stained with propidium iodide and flow cytometry was performed to separate cells based on DNA content. Graph shows the percentage increase of cells in each cell cycle phase versus the percentage of cells normally in that phase when cells are treated with DMSO vehicle control. B. Same was performed for 030 and 004 matching tumor lines. C. Primary normal and tumor cell lines were treated with either DMSO (D) or VMY-1-103 (V) for 18 hours. Western blot was performed for p21and p53 protein levels.

Figure 3: Knockdown of p53 in LNCaP cells greatly reduces VMY-1-103-mediated apoptosis

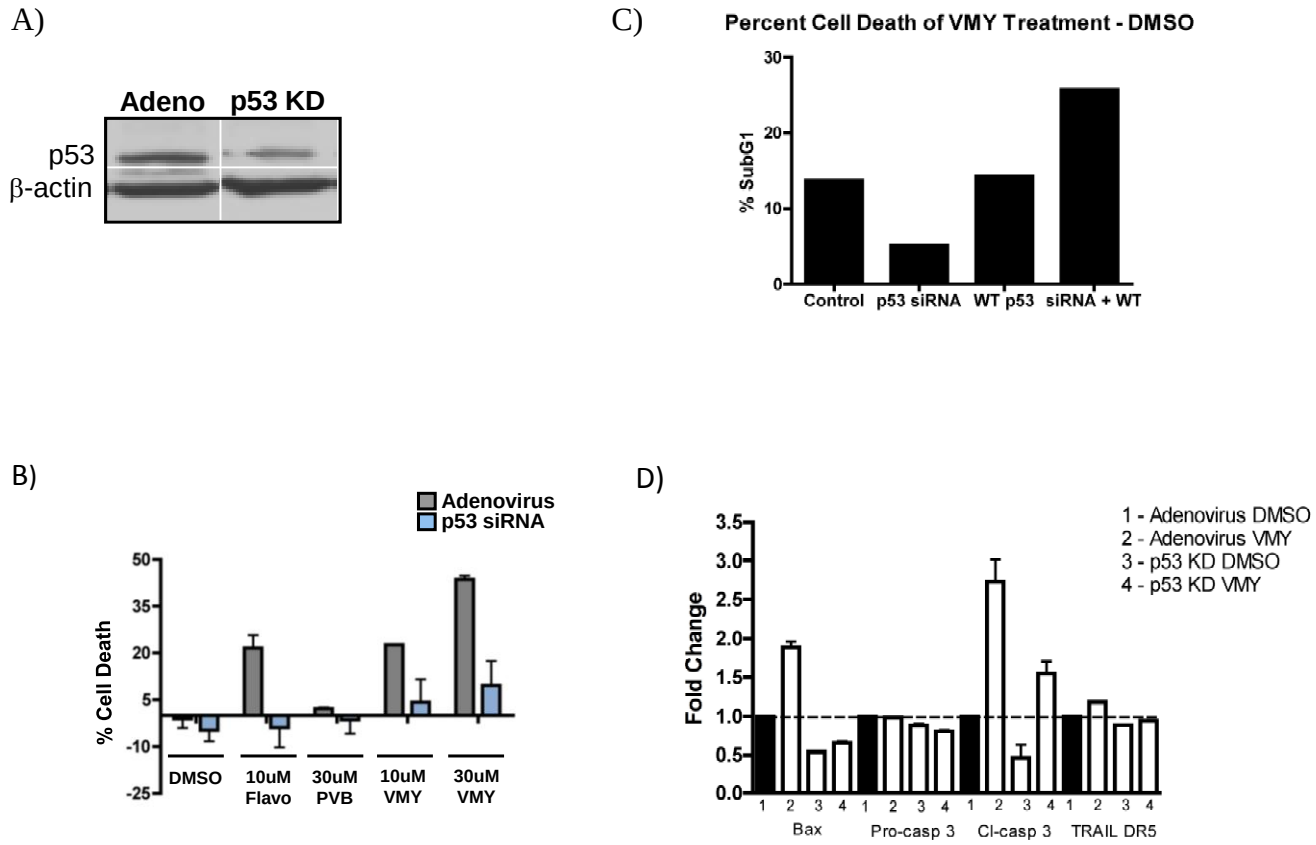

Figure 3: A) P53 protein levels in LNCaP cells decrease after addition of a p53 siRNA adenovirus for 72hr as compared to addition of adenovirus alone. B) LNCaP cells were treated with either adenovirus or p53 siRNA for 72hrs, followed by treatment with either flavopiridol (Flavo), purvalanol B (PVB), 10uM or 30uM VMY for 18hr. Cell death was quantified by trypan blue dye exclusion. C) LNCaP cells with and without p53 siRNA were transfected with a wild-type p53 vector, followed by treatment with either DMSO or VMY. Percentage of cell death was quantified by the subG1 fraction of cells using flow cytometry. The percentage of dead cells with DMSO treatment was subtracted from the percentage of dead cells after VMY treatment. D) VMY-1-103 treatment in the presence of adenovirus alone induce apoptosis as measured by an increase in Bax, cleaved Caspase 3, and TRAIL DR5 protein levels. These levels are reduced following p53 siRNA treatment.

Figure 4: Addition of wild-type p53 into p53 null PC3 cells greatly sensitizes them to VMY treatment.

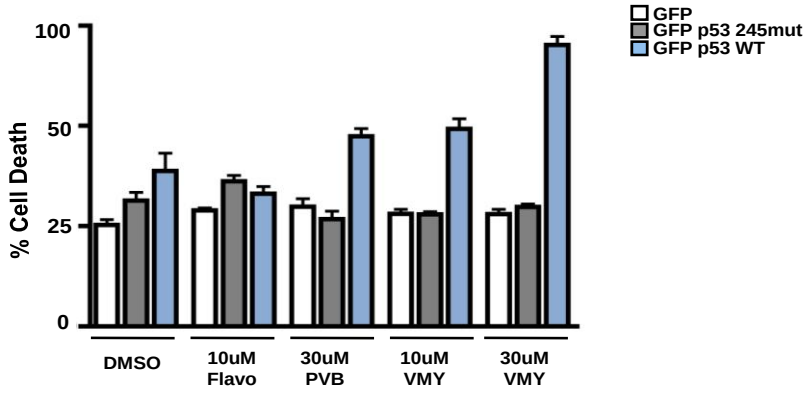

Figure 4: Transient transfection of either GFP, GFP-mutant 245 p53, or GFP-WT p53 constructs in PC3 cells followed by treatment with either DMSO, flavopiridol, purvalanol B (PVB), 10uM, or 30uM VMY. Percentage of cell death was measured by subG1 quantification using flow cytometry. Mutant p53 in addition to any drug treatment did not affect cell death, while WT p53 greatly increased cell death following purvalanol B and VMY treatment.

Figure 5: Treatment of p53-mutant DU145 cells with PRIMA-1 restores wild-type p53 function and sensitizes the cells to VMY.

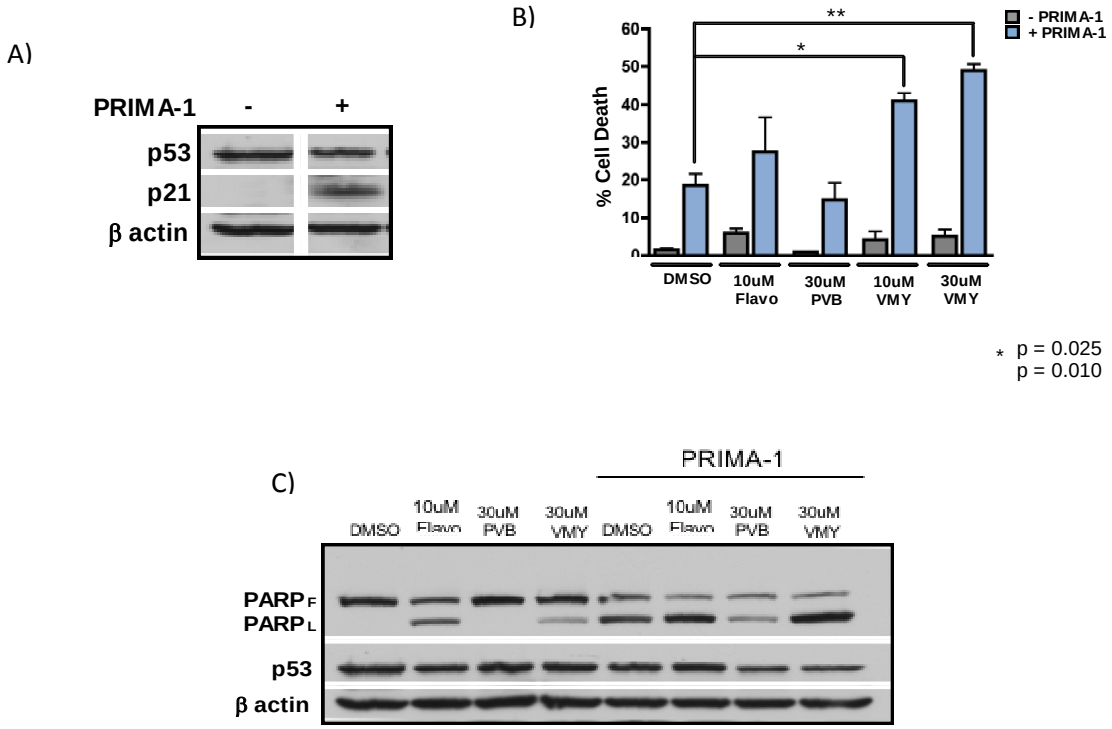

Figure 5: A) PRIMA-1 treatment in DU145 cells restores p53 as seen by induction of p21. B) PRIMA-1 pre-treatment of DU145 cells, followed by 18hr treatment with either flavopiridol, purvalanol B, or 10uM or 30uM VMY significantly increase the percentage of cell death as measured by trypan blue exclusion. C) PRIMA-1 treatment in DU145 cells increases levels of PARP cleavage following treatment with 30uM VMY.

Figure 6: Mutant p53 transfection in LNCaP cells does not effect knockdown of the wild-type protein.

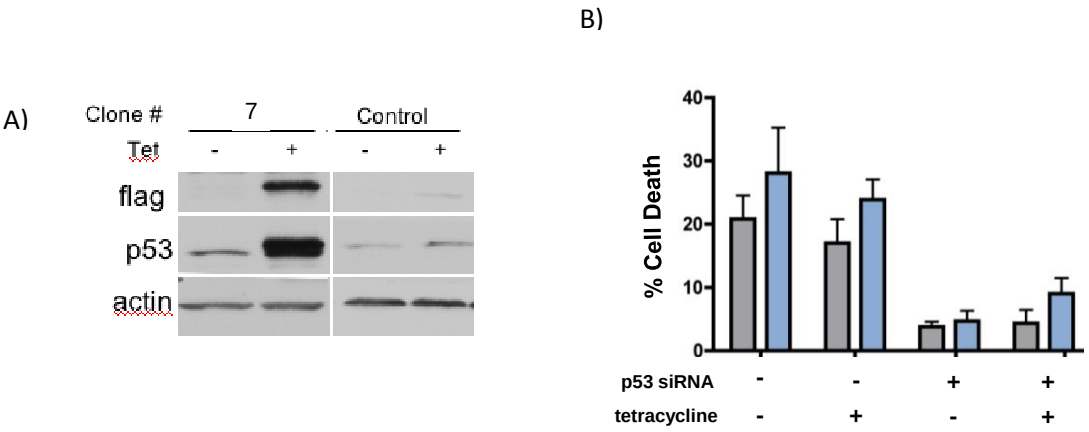

Figure 6: A) LNCaP cells were transfected with a tet-inducible p53 mutant (245) construct. Stable clones were selected using long-term G418 treatment and one representative clone is shown here. The construct is flag-tagged so flag protein and p53 levels increase upon addition of tetracycline in the stable population only. B) Percent cell death in LNCaP cells, as measured by subG1 content, increases upon 10uM and 30uM treatment versus DMSO treatment. Upon addition of p53 siRNA, VMY-mediated cell death is greatly reduced. Induction of the 245 mutant p53 construct via addition of tetracycline does not rescue the affect.

Figure 7: Inhibition of caspases 8 and 9 does not completely block VMY-mediated cell death.

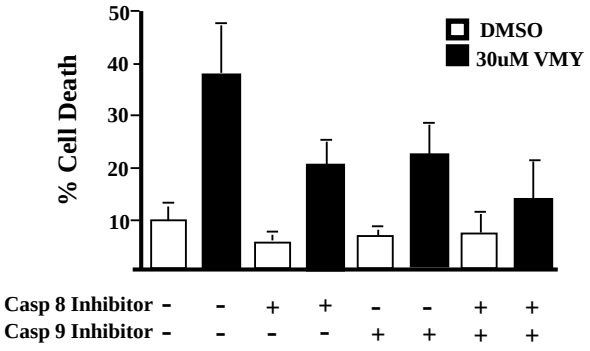

Figure 7: Cells were pre-treated with either the Z-IETD-FMK caspase 8 inhitibor, the Z-LEHD-FMK caspase 9 inhibitor, or both, followed by 18 hour treatment with either DMSO or 30uM VMY-1-103. Cell death was quantified by trypan blue exclusion.

Figure 8: VMY causes autophagy in LNCaP prostate cancer cells.

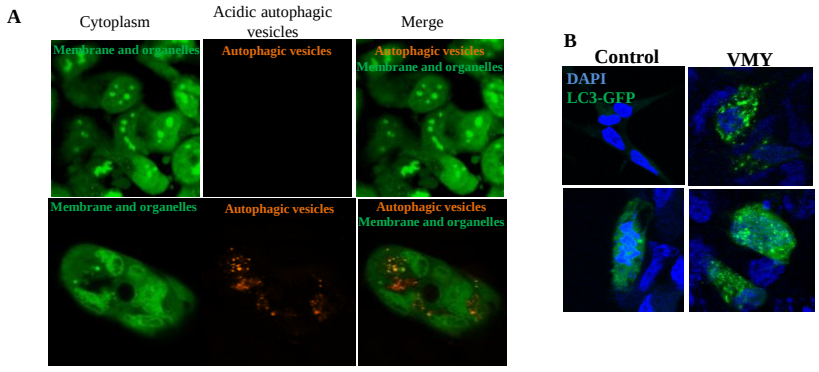

Figure 8: A. LNCaP cells were stained with acridine orange and treated with either DMSO (top) or VMY-1-103 (bottom). Cells were fixed and imaged under a confocal microscope. B. LNCaP cells were transiently transfected with an LC3-GFP construct and then treated with either DMSO or VMY-1-103. Cells were imaged using a confocal microscope.

Figure 9: The VMY-induced autophagic response in LNCaP cells is p53-dependent.

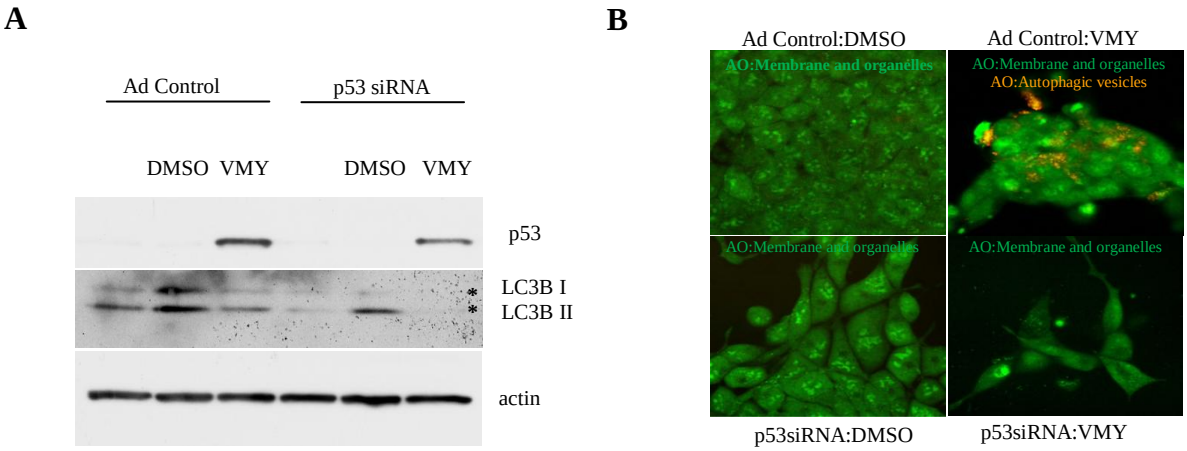

Figure 9: A) LNCaP cells were infected with either control adenovirus (Ad) or a p53 siRNA construct, followed by treatment with either DMSO or VMY-1-103. Western blot was performed for LC3BI and II products, as well as p53 levels. B) LNCaP cells were treated with either adenovirus control or a p53 siRNA construct, followed by DMSO or VMY-1-103 treatment. Cells were then treated with acridine orange (AO) and imaged using confocal microscopy.