The Response of p53 R249 Mutants to NSC319726 Treatment

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Review of Literature:

The p53 protein, encoded by the TP53 gene and comprised of 393 amino acids, was originally believed to be an oncogene, but was later determined to be a tumor suppressor gene whose role is crucial in the maintenance of an individual's genomic integrity (Baker et al., 1989). In fact, upon detecting the existence of cellular stress, such as the presence of an oncogene, the introduction of a chemotherapeutic drug, or the creation of DNA damage in a cell, p53 functions as a transcription factor, regulating the transcription of genes and proteins involved in numerous cellular processes important to cancer including apoptosis (programmed cell suicide), DNA repair, senescence (deterioration with age), and cell cycle arrest (Lin et al., 1995, Prives and Hall, 1999; Vousden and Lu, 2002; Riley et al., 2008; Vousden and Prives, 2009; Vousden and Ryan, 2009). Its applications vast and its duties necessary to ensure genomic stability, p53 has been referred to as the "cellular gatekeeper" and the "guardian of the genome" (Lane, 1992).

Given that the presence of too much p53 in a cell can result in cell death, p53 levels are known to be tightly regulated by p53's target genes. In fact, whereas p21 and PUMA are responsible maintaining genomic stability, taking charge of cell cycle arrest and apoptosis, respectively, MDM2 (an E3 ubiquitin ligase) keeps the levels of p53 in a cell in check by creating an autoregulatory negative feedback loop with the protein. Indeed, p53 transcribes MDM2, which then targets p53 for ubiquitin-dependent degradation so as to prevent an excess of p53 in a cell (Oren and Rotter, 2010).

Despite the extensive attempts to ensure that DNA is not mutated in the cell, the p53 protein is frequently mutated, causing the protein to lose its wild-type (non-mutated) structure

and function. In truth, p53 is mutated in about 50% of human cancer cases and has an abrogated pathway in the majority of the remaining instances (Hollstein et al., 1994). Furthermore, the most frequent type of mutation in p53 is a missense mutation, in which one nucleotide of the protein's sequence is replaced for another, causing a one amino acid difference in the protein sequence of the gene. The majority of the said missense mutations are located in the DNA- binding core domain of p53- the region of p53 in which the protein binds to specific DNA sequences to promote the transcription of its target genes. The missense mutations in the DNA- binding domain of p53 account for 1/3 of the protein's mutations, and six of the codons in this domain (R175, G245, R248, R249, R273, and R282) have been termed the hot spot codons of p53 due to the fact that they are mutated in the greatest frequency as compared to the other codons of the protein (Freed-Pastor and Prives, 2009). The missense mutations in the hot spot codons, and in all other codons of p53, fall into two major categories: DNA-contact (mutants in which proper binding to DNA is compromised) and conformational mutations (in which the wild-type structure of the protein is abrogated) (Cho et al., 1994). Regardless of the class in which the mutation falls, however, most mutated p53 proteins not only lose wild-type p53 function, but also have been shown to gain some properties associated with tumor promotion such as metastasis, which are commonly referred to as the "gain of function" phenotype (Dittmer et al., 1993).

Due to p53's essential role in tumor formation, and because mutant p53 is found in high concentrations in tumor cells, drug development has targeted the restoration of wild-type p53 structure and function in p53 mutants (reactivating mutant p53). The NSC319726 compound, for instance, has recently been identified as a compound that can reactivate mutant p53 by restoring

wild-type p53 structure and function in the R175H p53 mutant (Yu et al., 2012). In fact, to obtain its functions in the cell, the p53 protein coordinates a Zn^{2+} ion, which partakes in the wild-type structure of the protein (Cho et al., 1994). In order to restore p53 function in the R175H mutant, NSC319726 acts as a zinc metallochaperone, transporting a zinc ion to the correct location in the p53 protein.

Research has indicated that the R175H mutant of p53 is a zinc- binding mutant, a mutant that is not able to correctly bind zinc, thus inhibiting p53 from performing its functions in the cell. It is currently known that four amino acids (C176, H179, C238, C242) are bound directly to the one zinc ion in each p53 protein. It has also been shown that several of these mutants are also reactivated by NSC319726, thus supporting the conclusion that NSC319726 can reactivate p53 mis-sense mutants with impaired zinc binding. It not known just how many other mutants have impaired zinc binding but recent data indicates that other hotspot mutants such as the G245 mutant is reactivated by the drug (unpublished data).

Statement of Purpose:

Given the proximity of several other hotspot mutants to the G245 and R175 mutants, scientists questioned if any other hotspot mutants might also be targets of the drug. Of particular interest is the p53 codon 249, the 6th hotspot mutation site, which, when mutated, is a conformational mutation. However, given the distance of the codon to the zinc- binding domain in the p53 protein, it was hypothesized that mutants of this codon do not have impaired zinc binding and therefore are insenstive to this drug. By carrying out genotyping, a cell growth inhibition assay (MTS Assay), immunoflourescent staining, a gene expression assay (quantitative

PCR), as well as protein level measurement (Western Blot), scientists set out to confirm that wild-type p53 function would not be reactivated in p53 R249 mutants upon exposure to NSC319726, thus demonstrating that mutations in the 249 codon do not interfere with proper zinc binding, and indicating that conformational mutants of p53 do not necessarily play a role in zinc binding, as had been previously presumed.

Methods and Materials:

Cell lines

TOV112D, Hs700T, PLC/PRF5 and H460 cell lines are lab stock, purchased from American Type Culture Collection (ATCC).

Genotyping

While the genotype of the TOV112D cell line had already been confirmed in the lab, it was necessary to verify the genotype of the Hs700T and PLC/PRF5 cell lines, as well as that of the H460 (p53 wild-type) cell line, which was used as a control in this experiment. In order to carry out the genotyping, RNA was extracted from each of the cell lines using the Qiagen RNeasy kit (Qiagen, Valencia, CA, USA), and RT-PCR was performed using a Reverse Transcription kit (Applied BioSystems, CA), followed by PCR with primers F: 5'-GCCTGAGGTTGGCTCTGACTG-3' and R: 5'-GATTCTCTTCCTCTGTGCGC-3'. The sequencing primer used to determine the genotypes of the cell lines was F: 5'-GCCTGAGGTTGGCTCTGACTG-3'. To facilitate the interpretation of the data, the sequences were aligned using the website http://multalin.toulouse.inra.fr/multalin/.

MTS Assay

Five thousand cells (from three cell lines- TOV112D, PLC/PRF5, and Hs700T) in 100μL of culture per well were seeded in a 96-well plate for one day to reach 50-60% confluence, at which point the cells were treated with serial dilutions of the NSC319726 drug and incubated for three days. The MTS reagent (Promega, Madison, WI, USA) was then added to each of the wells and cell growth was measured by the Victor Plate reader instrument (PerkinElmer, Waltham, MA, USA) at OD_{490nm} (optical density 490 nm).

Immunofluorescent Staining

Cells from the TOV112D, Hs700T, and PLC/PRF5 cell lines were grown on cover slips in a six well plate. The cells were treated with NSC319726 for 6 hours and washed with cold PBS. The coverslips were fixed with 4% paraformaldehyde for ten minutes and permealized with 0.5% Triton X-100 for five minutes, followed by staining with conformation specific antibodies. The PAB1620 recognizes p53 proteins with a wild-type conformation, while the PAB240 antibody recognizes p53 proteins with mutant conformation. The coverslips were stained with 1:200 dilutions of the PAB240 antibody and 1:50 dilutions of the PAB1620 antibody overnight, followed by a forty minute incubation with the secondary antibody (goat anti-mouse IgG) the next day. The primary antibodies PAB1620 and PAB240 were purchased from EMD chemicals (Gibbstown, NJ, USA). Finally, immunofluorescent images were taken under fluorescent microscope.

RNA Extraction and Quantitative RT-PCR

RNA was extracted from the TOV112D, PLC/PRF5, and Hs700T cell lines as per the protocol in the Qiagen RNeasy Kit (Qiagen, Valencia, CA, USA). Then, quantitative RT-PCR (TaqMan gene expression assays) was performed according to the instruction of the manufacturer (Applied BioSciences, Carlsbad, CA, USA). The relative gene expression levels were normalized with β-actin.

Western Blot

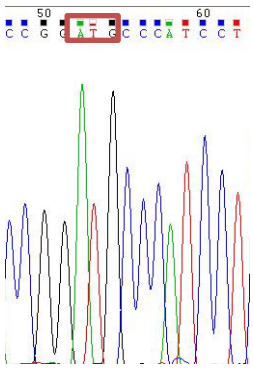
15μg of cell lysates from the TOV112D, Hs700T, and PLC/PRF5 cell lines were used to run an SDS-PAGE. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes and protein levels were measured according to the manufacturer's protocol (ECL, GE Healthcare, Waukesha, WI, USA). The p53 (DO-1) and actin antibodies were from Santa Cruz Biotechnology.

Results:

Genotyping Confirms the p53 Status of the Cell Lines

While it had already been confirmed in the lab that the TOV112D cell line was an R175H p53 mutant, it was necessary to verify that the PLC/PRF5 and Hs700T cell lines were codon 249 p53 mutants. With the H460 (wild-type p53) cell line acting as a control, the genotyping was performed. Initially, cDNA from the RNA extracted from the cells was subsequently amplified through PCR and sequenced. The area of interest for sequencing was codon 249 of Exon 7 (one of the sections of DNA that encodes the p53 protein). As shown in Figure 1, in the above-designated region of interest of DNA, the H460 cell line has the AGG genotype (which translates

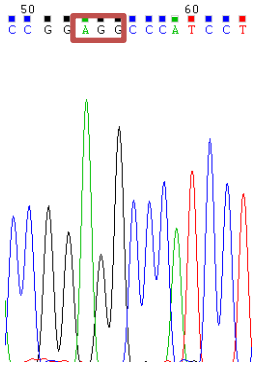
to the amino acid Arginine), whereas the PLC/PRF5 cell line has the AGT genotype (representing the amino acid Serine), and the Hs700T cell line has the ATG (amino acid Methionine) genotype. Thus, genotyping results confirmed that the p53 mutational status of PLC/PRF5 was R249S and that of Hs700T was R249M.



Hs700T Exon 7

Amino Acid 249 p53 **Mutant**

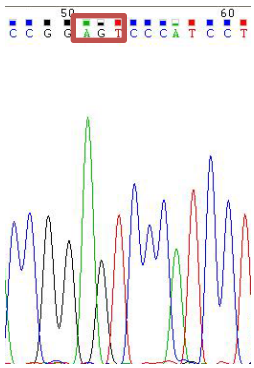
Amino Acid 249 Genotype: ATG Methionine **R249M**



H460 Exon 7

Wild Type p53

Amino Acid 249 Genotype: AGG Arginine



PLC/PRF5 Exon 7

Amino Acid 249 p53 **Mutant**

Amino Acid 249 Genotype: AGT Serine **R249S**

Figure 1. Genotyping Confirmed the Genotypes of the Hs700Tand PLC/PRF5 Cell Lines.

The Hs700T cell line was confirmed to be an R249M missense p53 mutant while the PLC/PRF5 cell line was confirmed to be a R249S missense p53 mutant.

NSC319726 does not Inhibit Cell Growth in R249 Mutants

An MTS Assay was used to test cell growth inhibition in the TOV112D, Hs700T, and PLC/PRF5 cell lines after NSC319726 drug treatment. As shown in Figure 2, the TOV112D

cells (as displayed by the red solid line) experienced cell growth inhibition after NSC319726 drug treatment, as published in (Yu et al., 2012), while the 249 mutant cell lines (portrayed by the solid and dotted green lines), despite slight fluctuation in cell survival percentage, remained quite stable after exposure to the drug. Furthermore, it was concluded that the concentration at which only 50% of the TOV112D cells were alive (the IC50) was 0.4 μM.

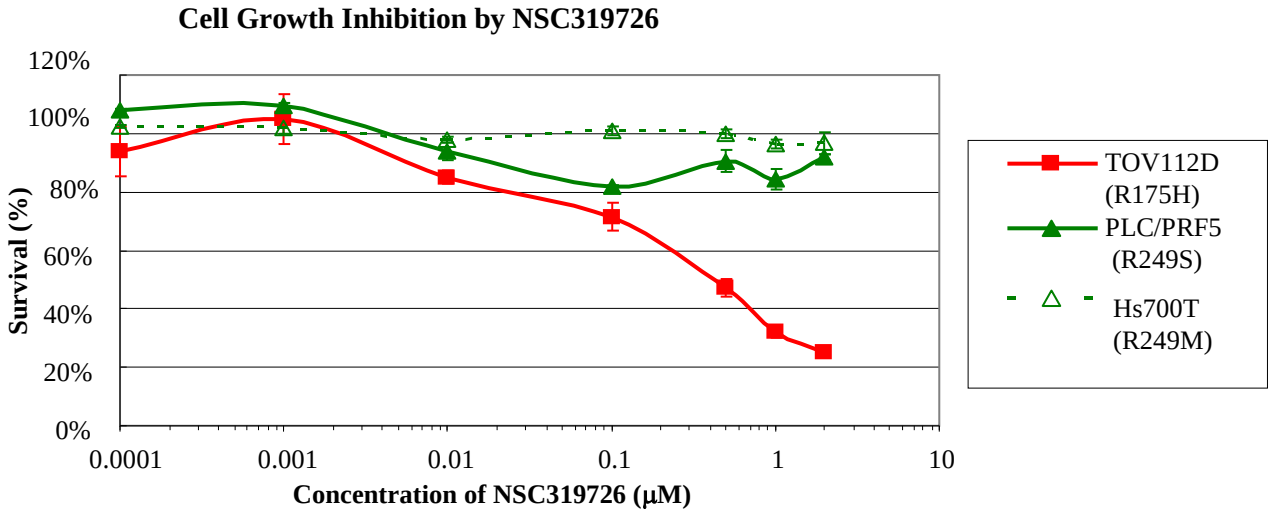


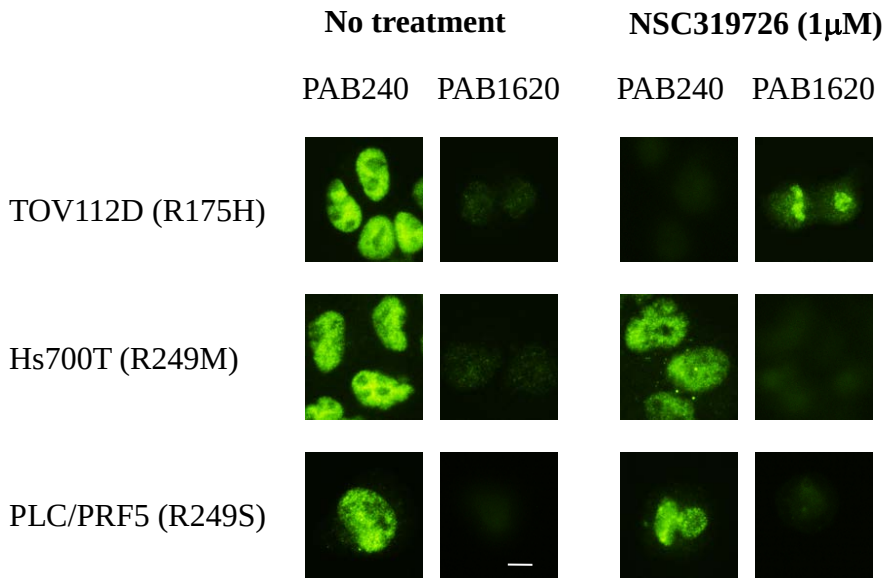
Figure 2. The Codon 249 p53 Missense Mutants are not Sensitive to NSC319726 Drug Treatment in terms of Growth Inhibition

Cells from three cell lines (TOV112D, Hs700T, and PLC/PRF5) were seeded at 5,000 cells/ well in 100 μL of medium in a 96- well plate and incubated for a day. Serial dilutions of NSC319726 were administered to the cells and the cells were incubated for three days, followed by MTS assay. The survival curve, indicating that the 249 mutants of p53 did not have growth inhibition after drug treatment, was then created.

NSC319726 Restores Wild-Type Conformation in R175H but not in R249 Mutant Proteins

Since the R175H, R249S and R249M p53 mutants are all conformational mutations (as shown in Figure 3, left panel, and explained below), and it is known that NSC319726 restores wild-type p53 activity to R175H mutants by acting as a zinc metallochaperone (Yu et al., 2012), experiments were carried out to examine whether or not R249 mutants responded to the drug in the same positive manner. While the PAB1620 antibody only recognizes the wild-type

conformation of p53, PAB240 only attaches to mutant p53 proteins. Thus, being that only the PAB240 antibody bound to p53 proteins in the TOV112D, Hs700T, and PLC/PRF5 cells before the addition of NSC319726, it was confirmed that all cell lines were conformational mutant p53 proteins (Figure 3 left panel). Post treatment, only p53 proteins in TOV112D cells were recognized by the PAB1620 antibody, thus indicating that wild-type p53 conformation was only restored in TOV112D cells, but not in the R249M (Hs700T) and R249S (PLC/PRF5) mutants (Figure 3, right panel).



The scale bar represents a size of 25 μm.

Figure 3. NSC319726 Does Not Induce a Wild-Type Conformation Change in the R249 p53 Missense Mutants.

The three mutant p53 cell lines (TOV112D, Hs700T, and PLC/PRF5) were treated with 1μM of NSC319726 for six hours and underwent immunofluorescent staining. The PAB240 antibody only recognizes the conformation of mutant p53 proteins while the PAB1620 antibody only binds to p53 proteins with a wild- type conformation. Because only PAB240 recognized p53 in TOV112D, Hs700T, and PLC/PRF5 cell lines before NSC319726 treatment, it was concluded that all cell lines had p53 with mutant conformations. After NSC319726 treatment, wild- type p53 conformation was only restored to TOV112D cells, as supported by the fact that the protein was now recognized by PAB1620, the wild- type conformation- specific antibody.

NSC319726 does not Restore p53 Transcriptional Function in R249 p53 Mutants

As a response to the presence of cellular stresses such as DNA damage or the manifestation of an oncogene in a cell, wild-type p53 will respond by attaching to the promoters of several genes and proteins, and, by doing so, will thus encourage their transcription. The genes and proteins transcribed by p53 are in turn responsible of such tasks as apoptosis (cell suicide), cell cycle arrest, and senescence. p21 and PUMA, responsible for cell cycle arrest and apoptosis, respectively, are the most commonly studied p53 target genes (Prives and Hall, 1999; Vousden and Lu, 2002; Riley et al., 2008; Vousden and Prives, 2009; Vousden and Ryan, 2009). As such, one of the most common methods of checking if p53 wild-type structure and function has been restored in a cell is to check the levels of p21 and PUMA in the cell. As shown in Figure 4, while p21 transcription was induced in TOV112D cells after NSC319726, this was not true for the 249 mutant cell lines, thus showing that p53 wild-type transcription ability had been restored to TOV112D cell lines but not to the 249 p53 mutant cells. Furthermore, PUMA transcription was induced in TOV112D but not in PLC/PRF5 cells. It was noted, however, that 24 hours after NSC319726 treatment, Hs700T cells had markedly increased PUMA levels, an observation that can only be explained by concluding that PUMA transactivation in this cell line was NSC319726-dependent, but p53- independent. Nevertheless, further experiments need to be conducted to clarify this conclusion.

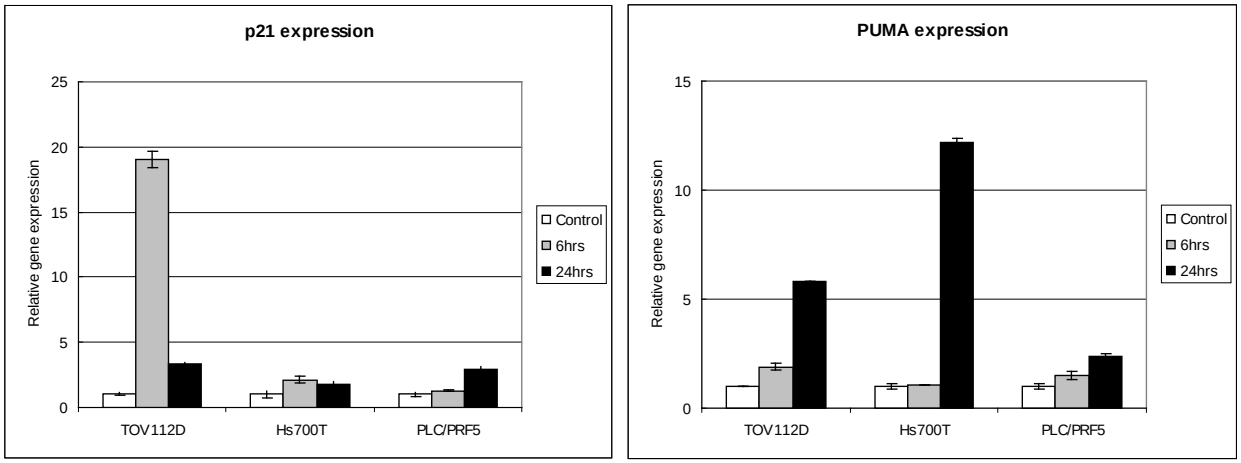


Figure 4. R249 p53 Missense Mutants Showed no Restoration of Wild- Type Transcriptional Activity Post NSC319726 Drug Treatment.
RNA was extracted from cells of the respective cell lines (TOV112D, Hs700T, and PLC/PRF5) and reverse- transcription followed by real-time PCR were performed so as to measure the gene expression level. It was found that whereas p21 expression was not induced in the Hs700T and PLC/PRF5 cell lines after NSC319726 treatment, PUMA was induced in the Hs700T cell line, primarily after 24 hours.

NSC319726 does not Change p53 Protein Levels (Stability) in R249 Mutants

Another commonly accepted means by which confirming that wild-type p53 activity has been restored in a cell is to analyze the concentration of p53 after an extended period of time. In fact, MDM2, an E3 ubiquitin ligase that targets p53 for ubiquitin-dependent degradation, is one of the target genes of wild-type p53. As a consequence, in its non-mutated form, p53 transcribes MDM2, which then degrades p53, creating a negative feedback loop (Oren and Rotter, 2010). As shown in Figure 5, due to the fact that the p53 protein level decreased six hours after drug treatment in TOV112D (R175H p53 mutant) cells, it can be suggested that p53 regained its wild-type transcriptional ability, and was able to transcribe MDM2, and thus be degraded. On the other hand, the p53 protein level in the Hs700T cell line did not decrease after six hours, indicating that p53 wild-type transcriptional ability had not been restored. Lastly, although p53 protein levels decreased in the PLC/PRF5 cell line after six hours, the same occurred in the actin

control. Being that actin levels normally remain stagnant in a cell over an extended period of time, and due to the fact that both p53 and actin levels decreased after six hours, the decrease in p53 protein level in this cell line is not significant or indicative of the restoration of wild-type transcriptional activity of p53.

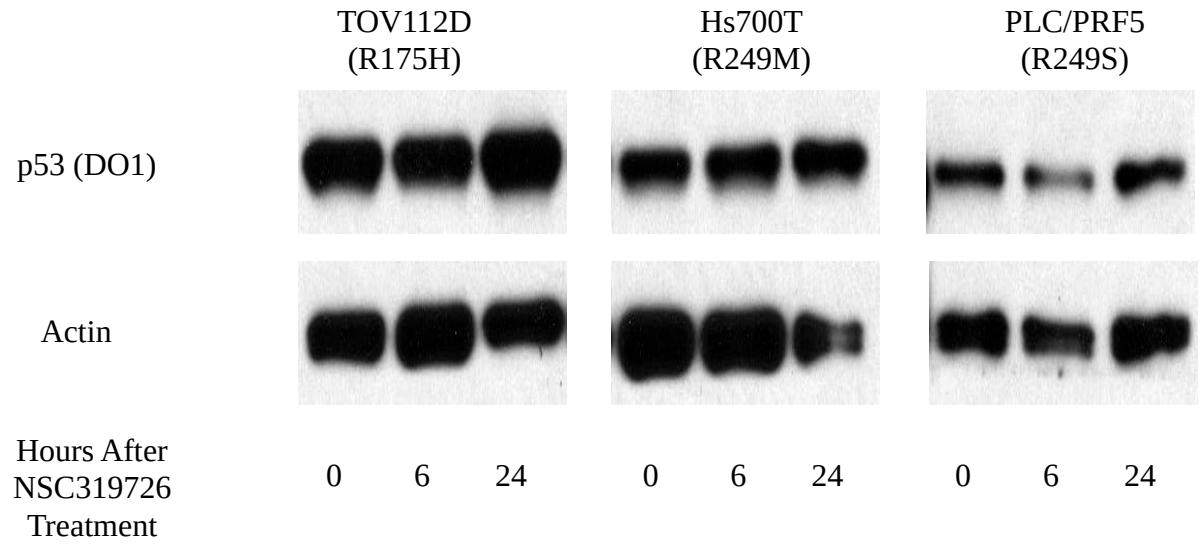


Figure 5. The Effects of NSC319726 Drug Treatment on R249 p53 Missense Mutant Protein Stability.

15 μg of cell lysates were loaded to SDS- PAGE and transferred to a PVDF membrane. The membrane, along with p53 (DO-1) and Actin antibodies were then used to perform a Western Blot. The decrease of p53 levels in the TOV112D cell line indicates that the ability of p53 to transcribe MDM2, a function only attributed to wild- type proteins, has been restored. Nonetheless, p53 levels in the Hs700T cell line did not decrease after NSC319726 treatment, indicating that wild- type transcriptional activity was not restored in this cell line, an observation that also holds true for the PLC/PRF5 cell line, which experienced a non-significant decrease in p53 protein levels as the actin control experienced the same pattern.

Discussion and Conclusion:

It has been known that, just as hemoglobin requires one Fe^{2+} ion to work properly, p53 coordinates one zinc ion so to attain its proper structure, and thus function (Cho et al., 1994). Codons of p53 known to be involved in zinc binding, such as the 175 codon, have had restored wild-type p53 structure and function upon treatment with NSC319726 because the drug acts as a

zinc metallochaperone, escorting a zinc ion to the necessary codons in p53 so as to ensure proper zinc binding and folding of the p53 protein (Yu et al., 2012). Due to the fact that the R249M (Hs700T cell line) and R249S (PLC/PRF5 cell line) p53 mutants are both conformational mutants, as indicated by the fact that the p53 in these cells are only recognized by the antibody that binds to p53 with a mutant conformation (PAB240), it seemed plausible that R249 p53 mutants would be reactivated by the NSC319726 drug and therefore that the 249 codon of p53 was involved in zinc binding. Nevertheless, data from the experiments detailed above suggest that the codon 249 of p53 is not involved in zinc binding. In fact, while it was confirmed that the TOV112D (R175H) cell line responded to the NSC319726 drug by exhibiting growth inhibition, a restoration of wild-type p53 transcriptional ability, and a transformation to the wild-type conformation of the protein, as had been previously discovered (Yu et al., 2012), it was found that the R249 p53 mutants did not respond to the drug in the same way. The Hs700T and PLC/PRF5 cell lines exhibited no cell growth inhibition upon exposure to NSC319726, and did not have reactivated wild-type p53 conformation or transcriptional ability post drug treatment. Because the 249 mutants of the p53 are not reactivated in any way by NSC319726, it can be concluded that due to the distance of the 249 codon of p53 from the region of the protein in which zinc binding occurs, it appeared not involved directly in zinc binding. As such, the 12-fold increase in PUMA expression in the Hs700T cell line 24 hours after drug treatment was quite surprising. Given that mutant p53 in R249 cells does not regain wild-type structure and function by NSC319726 treatment, the most logical conclusion to make concerning this outstanding data is that the transcription of PUMA in Hs700T cells is p53-independent, though further experiments must be conducted to clarify this conclusion.

NSC319726 has been proven to restore wild-type p53 structure and function in many hot spot mutants of the protein, such as the R175H p53 mutant (Yu et al., 2012). Furthermore, recent research at the Cancer Institute of New Jersey has been conducted to expand knowledge regarding the applications of the thiosemicarbazone NSC319726, thus concluding that three other hotspot mutants (C176F, C242S, G245S) also have restored wild-type p53 structure and function after exposure to the drug (unpublished data). Although the two codon 249 mutants tested in the experiments detailed above do not respond to NSC319726 to have restored wild-type p53 abilities, scientists are still adamant in their pursuit of a positive response to NSC319726, particularly in the hot spot codons of p53, of which the 249 codon is part. There is much hope that, in the near future, drug development and cancer therapy will be advanced to a point that personalized medicine (genotyping a patient so as to determine the mutations in the cancer cells and administer a drug to the patient to restore the function of the protein or gene in question) will become a realistic proposition.

References:

Baker, S., Fearon, E., Nigro, J., Hamilton, S., Preisinger, A., Jessup , J., VanTuinen, P., & Ledbetter, D. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science, 244(4901), 217-221.

Cho, Y., Gorina, S., Jeffrey, P. D., & Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: Understanding tumorigenic mutations. Science, 265, 346-355.

Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A.K., Moore, M., Finlay, C., and Levine, A.J. (1993). p53 gain of function mutations. Nature Genetetics, 4, 42-46.

Freed-Pastor, W. A., & Prives, C. (2009). Mutant p53: one name, many proteins. Genes & Development, 26, 1268-1286.

Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., and Harris, C.C. (1994). Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Research, 22, 3551-3555.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. Nature, 358, 15-16.

Lin, J., Wu, X., Chen, J., Chang, A., and Levine, A.J. (1995). Functions of the p53 protein in growth regulation and tumor suppression. Cold Spring Harbor Symposia on Quantitative Biology, LIX, 215-223.

Oren, M., & Rotter, V. (2010). Mutant p53 gain-of-function in cancer. Cold Spring Harbor Perspective Biology, 2.

Prives, C., & Hall, P. A. (1999). The p53 pathway. The Journal of Pathology, 187(1), 112-126.

Riley, T., Sontag, E., Chen, P., & Levine, A. (2008). Transcriptional control of human p53-regulated genes. National Review Molecular Cell Biology, 9, 402-412.

Vousden, K. H., & Lu, X. (2002). Live or let die: the cell's response to p53. National Review Cancer, 2(1), 594-604.

Vousden, K. H., & Prives, C. (2009). Blinded by the light: the growing complexity of p53. Cell, 137, 413-431.

Vousden, K. H., & Ryan, K. M. (2009). p53 and metabolism. National Review Cancer, 9, 691-700.

Yu, X., Vazquez, A., Levine, A. J., & Carpizo, D. R. (2012). Alle-specific p53 mutant reactivation. Cancer Cell, 21, 614-625.