JOURNAL OF
EMERGING INVESTIGATORS

Article

Investigating the potential of zinc oxide nanoparticles and zinc ions as promising approaches to lung cancer

Tannishtha Sethi[1], Donna Leonardi[1]
[1] Bergen County Academies, Hackensack, New Jersey

SUMMARY

Lung cancer is the leading cause of cancer casualties globally, with adverse side effects and drug resistance making current therapeutic options inadequate. Therefore, novel therapeutics require development. A major genetic contributor to these cancers is the mutant *TP53* gene, whose mutated protein fails to transactivate p21, a cell cycle control protein in p53-p21-RB signaling, resulting in uncontrolled cell growth and cisplatin resistance. Recent studies suggest zinc oxide nanoparticles (ZnO NPs) show promise as a targeted, cytotoxic therapy; however, their mechanisms and efficacy are strongly debated as they aggregate in solution, reducing bioavailability while demonstrating poor aqueous solubility. As such, we hypothesized that a combined treatment using either ZnO NPs and cisplatin or zinc ions and cisplatin would induce cancer cell apoptosis and reduce cisplatin resistance. We treated mutant p53 lung cancer cells with ZnO NPs, zinc ion solution isolated from the NP suspension, and/or cisplatin; all significantly reduced proliferation individually and in combination. Increased reactive oxygen species (ROS) and p21 upregulation from ZnO NP treatment suggests mechanisms involving p21, perhaps through a p53-independent mechanism or via p53 reactivation. Zinc ion solution rendered a reduction in p21 in the presence of significantly lower ROS induced by ZnO NP, suggesting a mechanism needing further elucidation. We subjected a control cell line, HaCaT cells, to the same treatments and demonstrated no significant reduction in proliferation in the presence of ZnO NPs or zinc ions. Our research suggests the use of ZnO NPs and zinc ions as potential strategies for lung cancer treatment regimens.

INTRODUCTION

Lung cancer is the leading cause of cancer related deaths across the globe, with over 1 million fatalities in 2020 alone (1). Lung cancer is a condition in which aberrant cells in the lungs divide uncontrollably, forming tumors that obstruct normal physiological function. The known causes of this disease include smoking, exposure to asbestos and radon, air pollution, and genetics. There are two major categories of lung cancer: Eighty-five percent present as non-small cell lung cancer (NSCLC), while the remaining 15% present as small cell lung cancer (SCLC), a more aggressive type of lung cancer (2). The most common type of NSCLC is lung adenocarcinoma; however, despite having treatments, the first-line approaches of surgery and chemotherapy have a 40% '1-year' survival rate (3). Despite advancements in chemotherapy, radiation therapy, and immunotherapy, therapeutic options for lung cancer have fallen behind due to their negative side effects (4). Failure of these treatments to produce a complete anti-cancer response due to the development of drug resistance or the inability to differentiate between cancerous and normal cells contributes to the high death toll of lung cancer (5). Therefore, novel therapeutic protocols must be developed to improve upon current treatment options.

A potential solution for the aforementioned problem would be to approach lung cancer in a targeted fashion on a molecular level. In essence, it would be necessary to analyze mutant genes and proteins which cause abnormal, uncontrolled cellular responses. One gene strongly associated with lung cancer and commonly present in many other cancers, is the mutated *TP53* gene (6). The wild-type *TP53* gene provides instructions for transcribing the tumor suppressor protein p53, often regarded as the "Guardian of the Genome," controlling abnormal cell cycle progression as well as the induction of apoptosis (7). When cancer-causing abnormalities are present in the DNA, the wild-type p53 protein activates the p53-p21 pathway, inducing cell cycle arrest through several cell cycle regulating proteins. When the mutated p53 gene produces a mutant p53 protein, p53 transcriptional control of p21 is inhibited and cell growth is not appropriately regulated. With the inability of mutant p53 to act as a trans-activator of p21, cell cycle progression becomes uncontrollable, potentially resulting in the formation of tumors (8). Therefore, a heterozygous or homozygous mutation of the p53 gene is a prominent issue that many researchers have attempted to address, thus far with limited success.

Interestingly, work by Ha *et al.* suggests that even though mutant p53 can be tumorigenetic, this mutant protein still has apoptotic potential in the presence of ZnO NPs through an unknown mechanism (9). Alternatively, work by Jung *et al.* suggests that the transcriptional upregulation of p21, an inhibitor of cyclin-dependent kinase complexes, can be independent of p53 transcriptional control, indicating there is an alternate mechanism leading to the increase of p21 protein expression (10).

Similarly, as with the work of Ha *et al.*, recent studies have shown that ZnO NPs themselves exhibit a high degree of cancer cell selectivity with the ability to surpass the therapeutic indices of some commonly used chemotherapeutic agents (11). Nanomedicine technologies have cleared the path for novel targeted cancer therapies by allowing therapeutic compounds to be encapsulated and delivered selectively to tumors via cancer cell surface antigens, passive permeation, and active internalization mechanisms (12). Employing

https://doi.org/10.59720/23-128

nanoparticles for therapeutic purposes has also been found to minimize resistance, addressing one of the most significant obstacles to conventional therapy (13). Therapeutic and diagnostic techniques based on nanotechnology have thus shown tremendous promise in improving cancer therapy in recent years (14).

However, a problem with the utilization of ZnO NPs has been that they are prone to clustering or aggregating in aqueous solution and are not representative of typical nanoparticle size (15). Furthermore, the aggregation influences the dissolution of the particle to zinc ions, as shown by Misra *et al.* (16). Accordingly, the scant investigations found in the literature regarding the use of ZnO NPs indicate controversy over the mechanism of toxicity (10). It remains unknown whether it is the ZnO NP aggregates or the dissolution to the zinc ion that confers cytotoxicity (17).

Importantly, cisplatin is a common chemotherapeutic treatment that has been employed in the treatment of NSCLC. However, cisplatin has toxic side effects at the concentration currently recommended and is responsible for nausea, cardiotoxicity, hepatotoxicity, nephrotoxicity, and neurotoxicity (18). Cisplatin has also been shown to be involved in the activation of mutant p53 and upregulation of p21, creating a pattern of senescence in lung cancer cells, which have damaged DNA (19). The upregulation of p21 via cisplatin plays an important role in regulating senescence in both dependent and independent pathways (20). Therefore, designing a drug delivery system that targets these cancer-causing genes exclusively while leaving healthy cells alone is imperative. In addition, reducing the dose of cisplatin required to show efficacy may minimize or completely alleviate the drug's deleterious side effects.

Our *in vitro* research used the lung cancer cell line CRL-5800, which is homozygous for a p53 missense mutation located at amino acid position 246 with the conversion of methionine to isoleucine (21). We hypothesized that a combinatorial treatment with ZnO NPs and cisplatin or zinc ions and cisplatin would reduce cancer cell proliferative potential and cisplatin resistance.

Our research also aims to understand the mechanism of any proposed cytotoxic effect, focusing on p53 dependent and independent mechanisms with emphasis on the protein expression of p21. One such mechanism includes reactive oxygen species (ROS), the result of unstable molecules containing reactive oxygen that readily interact with other molecules in the cell, disrupting homeostasis. The excessive buildup of ROS can damage DNA, RNA, and other significant molecules, and apoptosis and autophagic death can be activated in cancer cells through intrinsic and extrinsic mechanisms (22).

Additionally, the purpose of our study was to determine if the concentration of the first-line treatment for lung cancer, cisplatin, can be lowered to achieve lung cancer cell cytotoxicity *in vitro* when combined with ZnO NPs or zinc ions in solution, thereby potentially reducing the toxic side effects of cisplatin.

Through our research, we found that both ZnO NPs and zinc ions when working individually or in combination with cisplatin, resulted in reduced cell proliferation. Moreover, we discovered potential mechanisms for the differences in cell proliferation rates, but further elucidation of mechanisms is required. Therefore, alternative treatment options for this debilitating disease can be explored and potentially used to further the lifespan of the millions of people suffering from this disease.

RESULTS

Effect of ZnO NP Suspensions vs. Zinc Ion Solution on CRL-5800 Lung Cancer Cell Proliferation

We created suspensions of ZnO NPs and zinc ions at concentrations ranging from 0–62 µg/mL. Following this, we treated CRL-5800 lung cells with ZnO NP suspensions and zinc ion solutions and incubated for 48 hours. We subsequently measured proliferation rates utilizing an MTS assay (CellTiter96). Lung cancer cell proliferation decreased significantly when treated with both ZnO NPs and zinc ions, as indicated by a one-way ANOVA with a Tukey post hoc test. Interestingly, all concentrations of both ZnO NPs and zinc ions, except for 15 µg/mL of ZnO NPs, had statistically significant differences from their respective 0 µg/mL control treatment ($p < 0.05$) **(Figure 1)**. Further investigation to deermine cytotoxic mechanisms is hence warranted.

Elucidating Cytotoxic, Anti-proliferative Mechanisms of ZnO NP Suspension and the Zinc Ion Solution

We then performed experiments evaluating ROS, with the purpose of determining whether ZnO NPs and/or zinc ions are involved in the production of ROS as a means of reducing cell proliferation. To detect ROS production, we added H2O2 substrate solution to both lung cancer and keratinocyte cells, and then measured luminescence after incubating the plate. Treatment with the ZnO NPs led to a significantly greater production of ROS per cell than the zinc ion treatment ($p < 0.001$) **(Figure 2a)**. Through a competitive ELISA, p21 protein expression increased significantly in the presence of ZnO NPs, yet decreased significantly when cells were treated with zinc ions ($p < 0.05$, one-way ANOVA) **(Figure 2b)**. These results suggest the idea of differing mechanisms of potential cytotoxicity or a concentration effect resulting from the ZnO

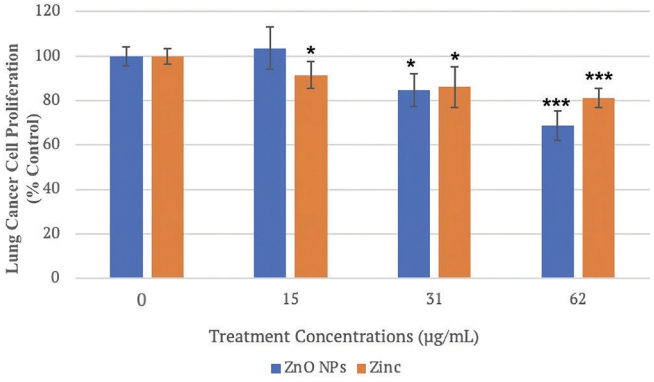

Figure 1: Determination of cell proliferation after ZnO NP vs zinc treatment. CRL-5800 lung cells were treated with ZnO NP suspensions and zinc ion solutions for 48 hours. ZnO NPs and the zinc ion exhibited a statistically significant difference in decreasing cell proliferation individually and across each concentration except the 15 µg/mL of ZnO NP. Bars are means ± standard deviation (n = 5). Statistical significance was indicated via one-way ANOVA with a Tukey post-hoc test and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to their respective 0 µg/mL control treatment (i.e., blue bars to blue 0 µg/mL bar and orange bars to orange 0 µg/mL bar).

https://doi.org/10.59720/23-128

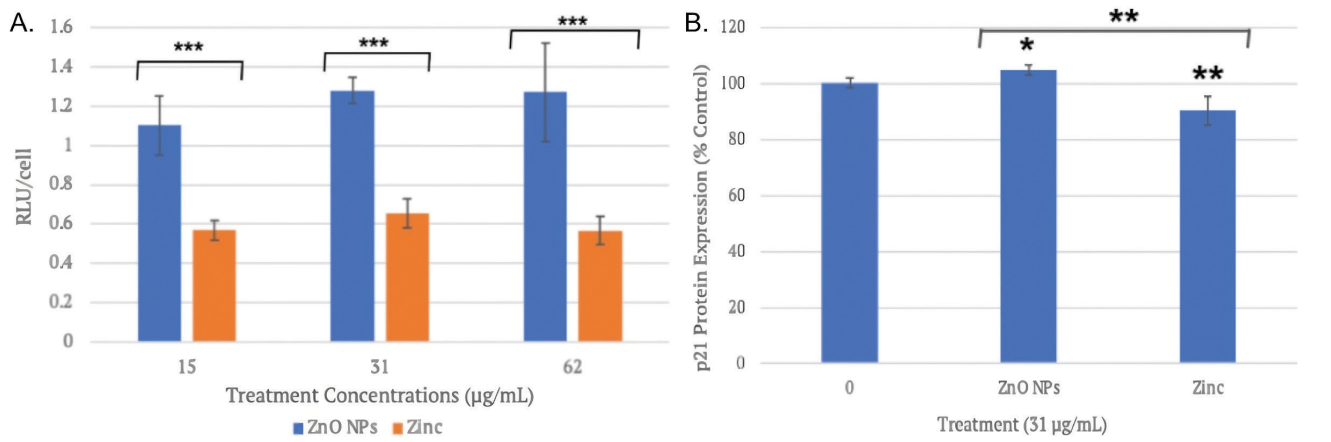

Figure 2: Determination of intracellular mechanisms of cytotoxicity. A) CRL-5800 lung adenocarcinoma cells were treated with ZnO NP suspensions and zinc ion solutions for 48 hours and ROS was measured via the Promega ROS-Glo H_2O_2 Assay (proprietary production of luciferase in the presence of ROS). RLU refers to the relative Luciferin luminescence units per cell, and the amount of H_2O_2 in the sample is directly proportional to the light signal. ZnO NP significantly increased ROS to a greater extent than zinc ion solution. Statistical significance was indicated through a two-way ANOVA with a Tukey post hoc test. Bars are means ± standard deviation (n = 5). Statistical significance was indicated through ***p < 0.001 when compared to each other. B) CRL-5800 lung adenocarcinoma cells were treated with a ZnO NP suspension and zinc ion solution of the same concentration, 31 µg/mL, for 48 hours and the protein expression of p21 was measured via an ELISA. There is an increase in p21 in the presence of ZnO NP and a decrease in p21 protein expression in zinc ion treated cells. This suggests alternate mechanisms. Bars are means ± standard deviation (n = 5). Statistical significance was indicated through a one-way ANOVA with a Tukey post hoc test and *p < 0.05 and **p < 0.01 when compared to the 0 µg/mL control treatment.

NP aggregation vs. the amount of zinc ion entering solution through dissolution.

Determining Treatment Effects on HaCaT Cell Line Proliferation

Parallelly, we conducted experiments on our control cell line, HaCaT. After preparing ZnO NP suspensions and zinc ion solutions, we administered them to the cells, incubating for 48 hours. Following the treatment period, we measured cell proliferation through an MTS assay (CellTiter96). The effect of both the ZnO NPs and the zinc ion on keratinocytes rendered no effect on proliferation **(Figure 3)**.

Analysis of Combinatorial Treatment Effects on CRL-5800 Lung Cancer Proliferation

For the combinatorial treatment experiment, we prepared ZnO NP suspensions and zinc ion solutions at concentrations ranging from 15–62 µg/mL. We then added a constant concentration of 25 µM cisplatin to the cells, along with the specific concentration of either ZnO NPs or zinc ions. When considering the effect of 25 µM cisplatin alone, where approximately 93% of the cells were left alive, all combinatorial treatments (regardless of nanoparticle, ion, or concentration) decreased the proliferation at a higher rate and the viability was always lower than 93% **(Figure 4a)**. The treatment for either the ZnO NPs or the zinc ion in combination with cisplatin resulted in a decrease in lung cancer cell proliferation across all concentrations in a dose-response fashion (p < 0.01) **(Figure 4b)**. Interestingly, when considering the ZnO NPs, the decrease in proliferation rate depicted a dose-response relationship. However, the zinc ion seemed to have an effect that plateaued at 31 µg/mL and 62 µg/mL. This data could also suggest different apoptotic mechanisms by the ZnO NPs as compared to the zinc ion when reducing viability in the cancer cells.

When examining the two combinatorial treatment regimens, the data demonstrates that the ZnO NPs and the zinc ion had significantly different effects on the proliferation at all concentrations. (p < 0.01) **(Figure 4b)**. This suggests varying mechanisms of cytotoxicity towards the lung cancer cells by treatment type. However, this may also be a concentration-dependent efffect since the exact concentration of zinc ion in solution is unknown. This reinforces the need to study the combinatorial treatment at a more versatile concentration range for the ZnO NPs and the zinc ion.

Considering the varying range of ZnO NPs and the zinc ion with the consistent cisplatin concentration of 25 µM, the combinatorial treatment seemed to exhibit an additive effect **(Table 1)**. Additive combinatorial treatments may suggest

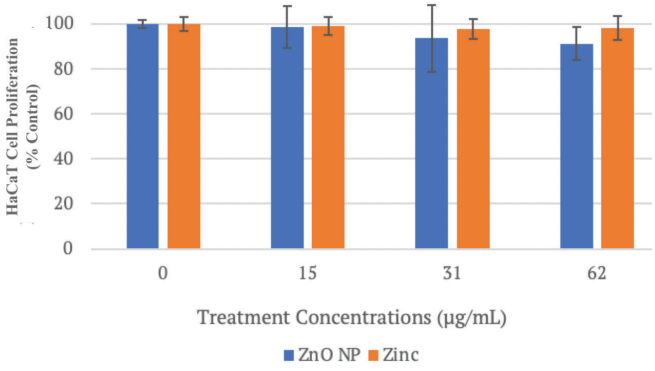

Figure 3: Determination of HaCaT cell proliferation after ZnO NP and zinc ion treatment for 48 hours. HaCaT cells were treated with ZnO NP suspensions and zinc ion solutions ranging from 0–62 µg/mL for 48 hours and proliferation was analyzed through an MTS Assay (CellTiter96). At the concentrations of ZnO NPs and zinc ions tested, there is no effect on proliferation. Bars are means ± standard deviation (n = 5). A lack of statistical significance indicates no significant decrease in viability of HaCaT cells after both treatments.

https://doi.org/10.59720/23-128

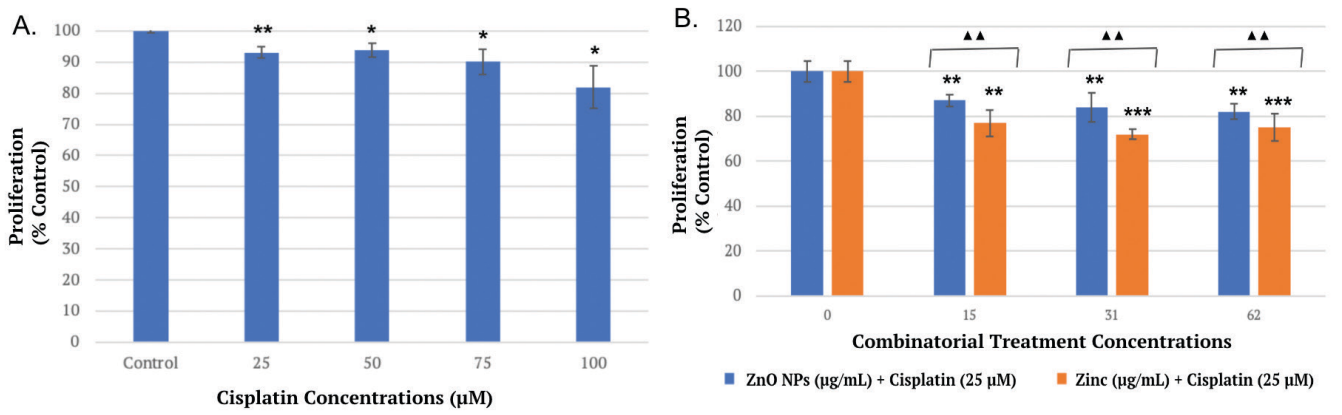

Figure 4: Determination of lung cancer cell proliferation after individual cisplatin treatment and combinatorial treatment of ZnO NP and cisplatin treatment or zinc ion and cisplatin treatment. A) Lung cancer cells with mutant p53 were treated with cisplatin solutions ranging from 0-100 μM for 48 hours and proliferation was analyzed via an MTS assay (CellTiter96). Cisplatin decreases proliferation in a dose-response fashion. Bars are means ± standard deviation (n = 5). Statistical significance indicated via a one-way ANOVA with a Tukey post-hoc test and $*p < 0.05$, $**p < 0.01$ when compared to solvent control (DMSO). B) Lung cancer cells with mutant p53 were treated with a cisplatin suspension of 25 μM and varying concentrations of ZnO NPs and zinc ion (μg/mL) for 48 hours and proliferation was analyzed through an MTS assay (CellTiter96). There is an antiproliferative effect with each treatment and a significant difference in effect between treatments across concentrations. Bars are means ± standard deviation (n = 5). Statistical significance indicated via a two-way ANOVA with a Tukey post-hoc test and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ when compared to their respective 0 μg/mL control treatment (i.e., blue bars to blue 0 μg/mL bar and orange bars to orange 0 μg/mL bar).
▲▲$p < 0.01$ when comparing between treatments at the indicated concentrations.

a similar molecular or cellular mechanism for the ZnO NP and zinc ion, contradicting the idea of alternate mechanisms suggested by other assays, thus warranting further research.

DISCUSSION

The original intention of our research study was to determine the potential of ZnO NPs as a promising anti-cancer regimen for lung cancer due to their reported selective cytotoxicity and ability to penetrate through physiological barriers. However, with the well-known aggregating effects of the ZnO NPs, along with their dissolution in aqueous solutions, we also incorporated an examination of the effects of both the ZnO NP suspension and the zinc ion into our study. Our investigation utilized a mutant p53 lung cancer cell line (CRL-5800) to address the difficulties in its treatment due to resistance to first-line therapeutics such as cisplatin. We used a keratinocyte cell line (HaCaT), a spontaneously immortalized cell line, as a control cell line in our study due to its derivation from normal adult skin and maintenance of a stable keratinocyte phenotype (23).

	Expected % reduction in proliferation	Expected % of control of proliferation	Actual % of control proliferation in combination treatment
Control	0%	100%	100%
Cisplatin (25 μM)	7%	93%	93%
Cisplatin (25 μM) + ZnO NPs (15 μg/mL)	7% + 0%	93%	87%
Cisplatin (25 μM) + ZnO NPs (31 μg/mL)	7% + 15%	78%	84%
Cisplatin (25 μM) + ZnO NPs (62 μg/mL)	7% + 30%	63%	82%
Cisplatin (25 μM) + Zinc (15 μg/mL)	7% + 10%	83%	77%
Cisplatin (25 μM) + Zinc (31 μg/mL)	7% + 15%	78%	72%
Cisplatin (25 μM) + Zinc (62 μg/mL)	7% + 20%	73%	75%

Table 1: Analyzing combinatorial treatment effect on lung cancer cells. Predictions for the expected reduction in proliferation were made by calculating the expected additive effects of Cisplatin 25 μM with the respective concentrations of ZnO NPs and zinc ion on CRL-5800 lung cancer cells. The ZnO NPs combinatorial treatment had an additive effect. However, the ZnO NP at 62 μM and 25 μM cisplatin showed a greater than additive reduction in proliferation than expected if additive (perhaps synergistic), warranting further research.

https://doi.org/10.59720/23-128

Our research first revealed that both ZnO NPs and zinc ions significantly reduced proliferation of lung cancer cells. Proliferation was evaluated utilizing an MTS assay, which measures the production of NADH. This correlates with the number of living cells only and is not indicative of dead or dying cells. A reduction in proliferation could indicate a reduction in living cells or slowing of population doubling time.

However, our data suggests ZnO NPs or zinc ions could be used in an anti-cancer regimen to treat mutant p53 lung cancer if research demonstrates the same findings *in vivo*. To provide deeper insight into the pathway mechanisms, caspase 8 and caspase 9, which have been shown to induce the "ultimate death enzyme" (caspase 3), through extrinsic and intrinsic pathways, respectively, should be measured. By doing so, it would be possible to determine whether the reduction in proliferation originates from an external factor (perhaps through ZnO NP membrane interactions) or inside the cell through intracellular signal transduction pathways. Additionally, the presence of the p21 protein produced by the mutant p53 lung cancer was analyzed as a result of the two treatments. Interestingly, the cells treated with the ZnO NP suspension indicated a significant upregulation of the p21 protein, while those treated with the zinc ion showed significantly less p21 protein expression compared to the control. This finding suggests that there are varying cytotoxic mechanisms induced by ZnO NPs and zinc ions in reducing proliferation.

Increased ROS, as well as p21 upregulation with ZnO NP treatments, suggests a mechanism involving p21, perhaps through a p53 independent mechanism or with p53 reactivation. Previous reports suggest that the presence of zinc has an effect in the refolding of the mutant p53 protein, leading to its reactivation (7). As the intracellular zinc ion increases, the metal spontaneously binds to mutant p53, restoring proper folding **(Figure 5)**. Regardless of the mechanism, upregulation of p21 effectively reduces cell cycle progression and can lead to apoptosis.

The zinc ion rendered a reduction in p21 in the presence of significantly lower ROS than ZnO NP, suggesting an alternate mechanism that is independent of p21 cell cycle arrest. The induction of autophagy by the zinc ion solutions would be an interesting hypothesis that could be the topic of future research. Perhaps the concentration of zinc ion in the solution may not be high enough to refold and reactivate mutant p53 as well. Several reported mechanisms of ZnO NP toxicity exist in the literature and require further elucidation **(Figure 6)**. Furthermore, it is important to note that while targeting is easier to achieve with the ZnO NPs, it is far easier to administer zinc ions than ZnO NPs *in vivo*.

With only a 5% increase in p21 protein expression due to the ZnO NPs and a 10% decrease due to the zinc ions, p21 may not be biologically relevant. While statistically significant, the role of p21 as a cytotoxic mechanism requires further investigation. To determine whether the effect of ZnO NPs or zinc ions is dependent on p21, p21 protein expression levels could be reduced via transfection with interference RNA. All previously measured parameters (proliferation, ROS, p21 levels, etc.) would be investigated in both the cells with constitutive amounts of p21 and the cells with the interference RNA.

Moreover, it is important to note that the concentrations mentioned throughout our research do not represent the actual ZnO NP or zinc ion concentrations in the cell culture but are named by dilutions of the stock of 12.5 mg/mL ZnO NP. Work by Reed *et al.* in 2012 suggests that ZnO NP dissolution rate to zinc ion is variable even between the different cell

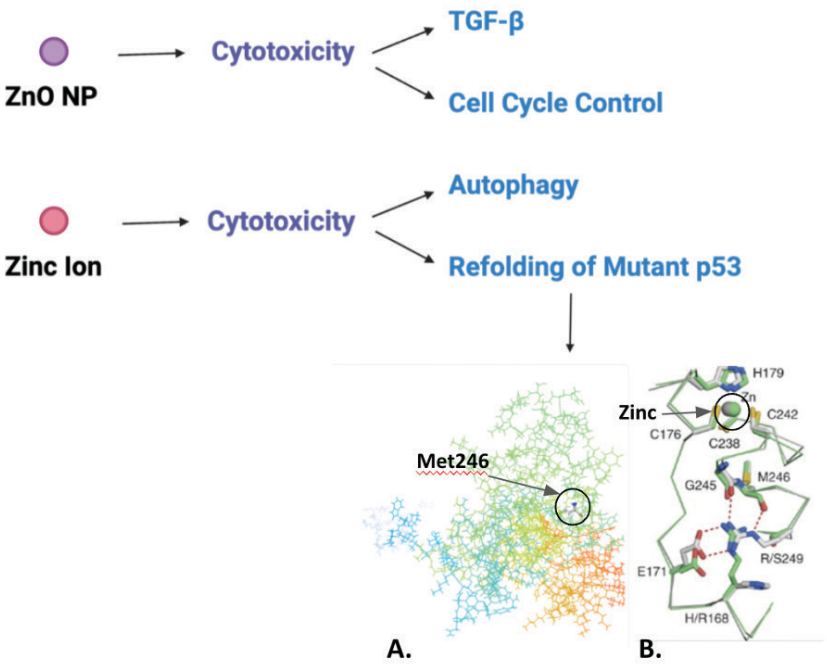

Figure 5: Further identification of cytotoxic mechanisms. A. wild type p53 protein demonstrating location of Met-246. Image created on SWISS-MODEL. B. Met-246 in its buried location within a hydrophobic pocket of the zinc-binding region.

https://doi.org/10.59720/23-128

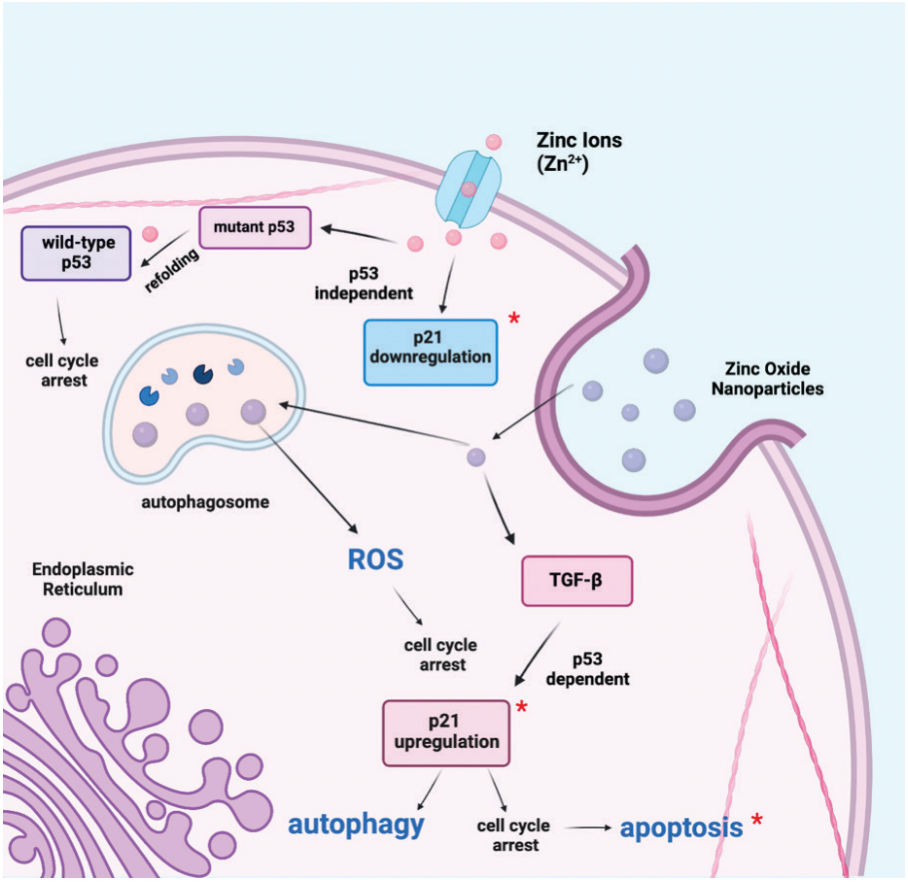

Figure 6: Mechanistic Proposal of Zinc Oxide Nanoparticles and zinc ions *in vitro*. This diagram demonstrates the potential of ZnO NP-cytotoxicity through various mechanisms reported in literature as well as novel findings reported by our research (indicated via a red asterisk). ZnO NPs and zinc ions enter the cell via multiple routes, including phagocytosis and ion channels. ROS and autophagy occur due to the zinc ions, toxicity of the nanoparticles, and the p53 and caspase-9 pathways. The zinc ion has also been reported to refold mutant p53 into a functional p53 through an unknown mechanism. TGF-β plays a role in p21 upregulation independent of p53, along with the ROS induced by the nanoparticles. Image adapted from Liu *et al.* and modified by author on BioRender (29).

culture media, ranging from a solubility of 5 mg/L to 34 mg/L (24). Throughout our paper, the concentrations listed on the graphs simply represent the dilutions of the 12.5 mg/mL stock concentration of ZnO NP and are termed "ZnO NP" and "Zinc," respectively **(Figure 7)**. In the future, experiments could be conducted to measure the exact amount of zinc ion present in the solutions.

It would also be of particular interest to measure the ROS in an untreated condition on the lung cancer cells to determine if either or both treatments increased or decreased levels of ROS compared to untreated control. There is controversy in the literature regarding the effect of ZnO NPs, which have been shown to significantly induce intracellular ROS while simultaneously exhibiting moderate antioxidant activity (22, 25). Studies have also indicated the ability of zinc ions to act as an antioxidant, therefore supporting the results of significantly higher induction of ROS due to ZnO NPs as compared to zinc ions (26, 27).

Data from the cisplatin experiments suggests that lowering cisplatin concentrations is necessary, as the drug doses which are cytotoxic to cancer cells (25 μM) are also cytotoxic to the keratinocytes. Thus, targeted nanoparticle treatments must be developed in order for the nanoparticles to be targeted solely to the cancer cells. In addition, perhaps the cisplatin concentrations can be lowered with a concomitant increase in the ZnO NP or zinc ion concentration, which may lead to more toxicity brought about by higher zinc.

However, the combinatorial treatment revealed interesting findings. Both the ZnO NP and zinc ion treatments in combination with cisplatin suggested an overall additive effect **(Figure 4, Table 1)**. Additive effects can occur when the molecules involved are in different signaling pathways and their simultaneous administration results in perturbing homeostasis through multiple avenues, thus supporting the data collected and analyzed in our study.

Further studies could be conducted to analyze and compare intracellular mechanisms of potential cytotoxicity, such as autophagy, between the ZnO NPs and the zinc ion. TGF-β protein could also be measured via an ELISA to analyze the connection with p21 (28). The idea that zinc can refold and reactivate p53, which is mutant in the cell line used in our study, must be studied by NMR spectroscopy or by crystallization and X-ray diffraction.

While the controversy of the ZnO NPs vs. zinc ions inducing cytotoxicity in lung cancer cells has been made clearer since both treatments result in a reduction of proliferation of the cells, further elucidation of mechanisms is required. As such, our study proposes an alternate approach to a nanoparticle

https://doi.org/10.59720/23-128

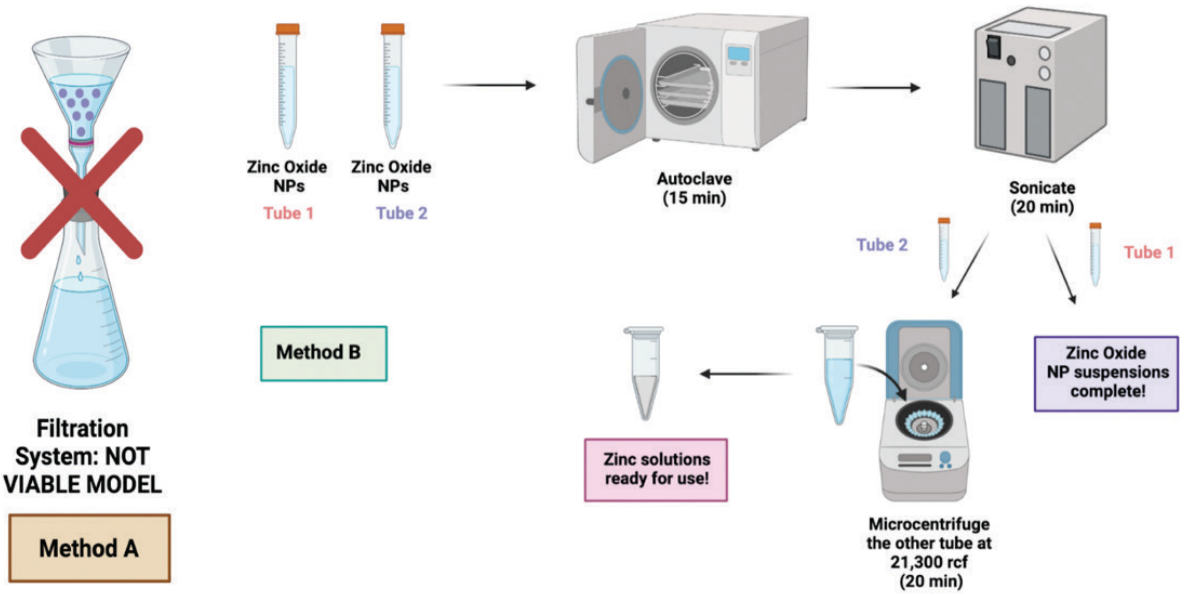

Figure 7: ZnO NP suspension and zinc solution preparation. Method A: Difficulty with ZnO nanoparticles being filtered through a 0.45 µm filter suggested that aggregates of ZnO particles were obstructing the filter. No ZnO nanoparticles were found in the minimal filtrate by TEM. This method was abandoned. **Method B:** Two ZnO nanoparticle suspensions in separate tubes with 12.5 mg/mL ZnO NP were autoclaved and sonicated. One suspension was now ready for use and was labeled "ZnO NP." The other suspension was microcentrifuged at 21,300 rcf (20 min) and the supernatant was serially diluted in water for treatment and named "zinc" or "zinc ion." Cells were then treated within one hour of completion of treatment preparation. Image created by author on BioRender.

that could be designed to selectively target and kill the lung cancer cells without inducing apoptosis in the non-cancerous cells, such as the keratinocytes used here. The ZnO NP could be coated with hydrophilic cisplatin and an antibody designed to target a cancer cell-surface antigen. This could then be administered into the bloodstream. The effect of this treatment would be the targeted death of the lung cancer cell.

Most importantly, our research indicates that a targeted nanoparticle treatment for mutant p53 lung cancer cells has enormous potential. We aimed to find selectively-targeting treatments to reduce proliferation of lung cancer cells that can eventually mitigate the side effects several patients face. We looked at the gene pathways affected on a more molecular level, analyzing mechanisms of cytotoxicity on a cellular level. Ultimately, if cisplatin concentrations can be lowered through a combinatorial approach, and the nanoparticles can be targeted, an optimal treatment can be created, impacting the lives of millions of people who suffer from this disease each year.

MATERIALS AND METHODS

Cell Culture

CRL-5800 (ATCC) and CRL-5872 (AddexBio), human lung adenocarcinoma cells and HaCaT keratinocytes, were maintained in RPMI and DMEM supplemented with 10% fetal bovine serum (ThermoFisher) respectively. Incubation conditions for cells were 37°C, 5% CO2, and 100% humidity. The cells were subcultured with 0.05% trypsin EDTA (ThermoFisher) once at 80% confluency.

Cell Culture

The cultured cells were plated in a 96-well plate, and treatments were administered for 48-hour incubation. Following incubation, 15 µL of MTS based Celltiter® 96 Aqueous One Solution Reagent (Promega, Madison, WI) was added to each well. The plate was then incubated for 1–2 hours. Using a SynergyHT Microplate Reader (BioTek, Winooski, VT), absorbance was read at 490 nm.

Preparation of Lysates

In accordance with treatment protocols, lysis buffer (Cell Signaling Technology, Danvers, MA, USA), phosphate buffered saline (Gibco, Waltham, Massachusetts, USA), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were placed on ice. Cells were then trypsinized and neutralized with RPMI, and 500 µL of the cells were counted on Vi-Cell XR Viability Analyzer (Beckman-Coulter, Indianapolis, IN). The remaining cells were centrifuged to pellet and spun at 1000 rpm for 7 minutes. The supernatant was decanted, and the pellet was resuspended in 2 mL of cold phosphate buffered saline. The supernatant was respun at 1,000 rpm for 7 minutes to re-pellet. PBS was then removed. Per Vi-Cell XR count, for every 1 x 106 cells in the pellet, 1 mL of 1x lysis buffer was added. For each 1 mL of lysis buffer, 10 µL of protease inhibitor cocktail was added for 10 minutes on ice. After the protease inhibitor cocktail was added, cells rested on ice for 10 minutes and transported into microcentrifuge and centrifuged for 15 minutes at 13,000 rpm. The supernatant was transferred to a pre-chilled microcentrifuge tube and samples were then kept in a -80 ºC freezer until needed.

ELISA: Indirect Enzyme-Linked Immunosorbent Assay

After lysates were plated in a 96-well ELISA plate for 24 hours at 4 ºC and subsequently removed, 300 µL of

https://doi.org/10.59720/23-128

BSA Blocking Buffer was added to each well for 10-minute incubation at room temperature. The primary antibody for p21 (Proteintech, Rosemont, IL, 10355-1-AP) was then prepared and added at 100 µL/well and incubated for 1 hour. After this, the goat anti-rabbit secondary antibody was prepared and added at 100 µL/well and then incubated for 1–2 hours. After adding 100 µL ABTS Substrate Solution (KPL ELISA kit, Seracare, Milford, MA) to each well, absorbance was measured at 405 nm. All ELISAs were controlled for direct comparison by producing lysates at 1,000,000 cells/mL.

ROS-Glo H_2O_2 Assay

After cells were plated in a 96-well plate for 24 hours, ZnO NP or zinc treatments were administered for 48-hour incubation. Following incubation, H_2O_2 substrate solution was prepared in accordance with Promega treatment protocols. Subsequently, 20 µL of prepared H_2O_2 substrate solution (Promega, Madison, WI) was added to each well and the plate was incubated for 6 hours. A detection solution was then added for 20 minutes, and luminescence (due to luciferase production) was read using a SynergyHT Microplate Reader (BioTek, Winooski, VT).

Preparation of ZnO NP Reagents for Cell Culture Treatment (Figure 7)

Suspensions of ZnO NPs of size 30 nm (Inframat Advanced Materials, Manchester, CT, 30N-0801) were prepared in sterile distilled water (ThermoFisher). To ensure sterility, suspensions were autoclaved for 15 minutes at 121 ºC. To disperse the aggregates, the suspensions were sonicated for 20 minutes. One of the stock suspensions was then labeled "ZnO NP," making it ready for use. The remaining stock suspension was then centrifuged at 21,000 rcf (20 minutes), and the supernatant was labeled "Zinc" or "Zinc ion." Following treatment creation, dilutions were prepared in sterile water to achieve desired concentrations in cell culture (0–62 µg/mL). Note that when 1 µL of dilution was added to 100 µL of cell culture the final concentration in cell culture was 1/100 dilution. Prior to treatment, cells were seeded for 24 hours and subsequently treated with dilutions (1:100) within 1 hour of preparation for an additional 48 hours.

Zinc Treatment Concentration Nomenclature

Treatments were named by dilutions of the original 12.5 mg/mL ZnO NP stock, without consideration of dissolution of the particles and aggregates. Concentrations were created by serial dilution, but lack of equipment such as Dynamic Light Scattering (DLS) capabilities and zinc ion measuring capabilities did not allow for exact quantification of particles or ions in solution.

Cisplatin Treatment Preparation

A stock solution of 3 mg/mL of cisplatin (Sigma-Aldrich, St. Louis, Missouri) was created in sterile DMSO (VWR BDH Chemicals, Radnor, Pennsylvania), and subsequent dilutions (in sterile DMSO) were created to achieve desired concentrations in cell culture. Note that when 1 µL of dilution was added to 100 µL of cell culture, the final concentration in cell culture was 1/100 dilution. Prior to treatment, cells were seeded for 24 hours, and subsequently treated with the dilutions (1:100) within 1 hour of preparation for an additional 48 hours.

Statistical Analysis and Significance

Assays were repeated three or more times unless noted with "n" greater than or equal to five. Data was normalized by converting absorbance values to percent of control per cell, using proliferation data or standardized lysing at 1,000,000 cells/mL for ELISA. Data analysis was performed using Microsoft Excel. A Student's *t*-test, one-way ANOVA, and two-way ANOVA, with a post hoc Tukey Test were performed to determine statistical significance ($\alpha = 0.05$).

Received: June 12, 2023
Accepted: August 18, 2023
Published: February 2, 2024

REFERENCES

1. Ferlay, J., *et al.* "Global Cancer Observatory: Cancer Today." *International Agency for Research on Cancer,* 2020
2. "What is lung cancer?: Types of lung cancer." *American Cancer Society,* 2019
3. Bai, K., *et al.* "Human lung adenocarcinoma cells with an EGFR mutation are sensitive to non-autophagic cell death induced by zinc oxide and aluminum doped zinc oxide nanoparticles." *The Journal of Toxicological Sciences,* vol. 42, 2017, pp. 437-450. https://doi.org/10.2131/jts.42.437.
4. Lahiri, Aritraa, *et al.* "Lung cancer immunotherapy: progress, pitfalls, and promises." Molecular cancer, vol. 22, no. 40, 2023, https://doi.org/10.1186/s12943-023-01740-y.
5. Mansoori, B., *et al.* "The Different Mechanisms of Cancer Drug Resistance: A Brief Review." *Advanced Pharmaceutical Bulletin,* https://doi.org/10.15171/apb.2017.041.
6. Canale, M., *et al.* "The Role of TP53 Mutations in EGFR-Mutated Non-Small-Cell Lung Cancer: Clinical Significance and Implications for Therapy." *Cancers,* vol. 14, 2022, no. 5 https://doi.org/10.3390/cancers14051143
7. Einstein, M. "A visual guide to restoring the guardian of the genome." Nature, 2022, https://doi.org/10.1038/d41586-022-00566-w.
8. Rivlin, N., *et al.* "Mutations in the p53 tumor suppressor gene: Important milestones at the various steps of tumorigenesis." Genes & Cancer, vol. 2, 2011, no. 4, pp. 466-474. https://doi.org/10.1177/1947601911408889.
9. Ha, J. H., *et al.* "p53 and Zinc: A Malleable Relationship." Frontiers in Molecular Biosciences, 2022, https://doi.org/10.3389/fmolb.2022.895887.
10. Jung, D., *et al.* "Quinacrine upregulates p21/p27 independent of p53 through autophagy-mediated downregulation of p62-Skp2 axis in ovarian cancer." Scientific Reports, vol. 8, 2018, https://doi.org/10.1038/s41598-018-20531-w.
11. Master, A. M., *et al.* "EGF receptor-targeted nanocarriers for enhanced cancer treatment." *Nanomedicine,* https://doi.org/10.2217/nnm.12.160.
12. Gavas, S., *et al.* "Nanoparticles for Cancer Therapy: Current Progress and Challenges." *Nanoscale Research Letters,* https://doi.org/10.1186/s11671-021-03628-6.
13. Alshehri, S., *et al.* "Progress of Cancer Nanotechnology as Diagnostics, Therapeutics, and Theranostics Nanomedicine: Preclinical Promise and Translational

https://doi.org/10.59720/23-128

Challenges." *Pharmaceutics,* https://doi.org/10.3390/pharmaceutics13010024.
14. Dessale, M., *et al.* "Nanotechnology: A Promising Approach for Cancer Diagnosis, Therapeutics and Theragnosis." *International Journal of Nanomedicine*, vol. 17, 2022, pp. 3735-3749. https://doi.org/10.2147/IJN.S378074
15. Sirelkhatim, A., *et al.* "Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism." N*ano-micro letters* vol. 7, 2015, no. 3 pp. 219-242. https://doi.org/10.1007/s40820-015-0040-x.
16. Misra, S. K., *et al.* "The complexity of nanoparticle dissolution and its importance in nanotoxicological studies." *The Science of the Total Environment,* vol. 438, 2012 pp. 225–232, https://doi.org/10.1016/j.scitotenv.2012.08.066.
17. Zhu, X., *et al.* "The impact of ZnO nanoparticle aggregates on the embryonic development of zebrafish (Danio rerio)." *Nanotechnology,* vol. 20, no. 19, 2009, https://doi.org/10.1088/0957-4484/20/19/195103.
18. Dasari, S., *et al.* "Cisplatin in cancer therapy: molecular mechanisms of action." *European Journal of Pharmacology,* vol. 740, 2014, pp. 364-378. https://doi.org/10.1016/j.ejphar.2014.07.025.
19. Takahashi, T., *et al.* "Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions." *Cancer Research,* vol. 52, no. 8, 1992, pp. 2340-2343.
20. Sriratanasak, N., *et al.* "Cisplatin induces senescent lung cancer cell-mediated STEMNESS induction via GRP78/AKT-dependent mechanism." *Biomedicines,* vol. 10, no. 11, 2022, https://doi.org/10.3390/biomedicines10112703.
21. Benavides, A., *et al.* "Methionine inhibits cellular growth dependent on the p53 status of cells." *American Journal of Surgery,* vol. 193, no. 2, 2007, pp. 274-83. https://doi.org/10.1016/j.amjsurg.2006.07.016.
22. Nakamura, Hajime, and Kohichi Takada. "Reactive oxygen species in cancer: Current findings and future directions." Cancer Science vol. 112, no. 10, 2021, pp. 3945-3952. https://doi.org/10.1111/cas.15068.
23. "HaCaT Cell Line (Human Keratinocytes)." *Antibody Research Corporation.* (n.d). https://antibodyresearch.com/HaCaT-cell-line-human-keratinocytes/
24. Reed, R. B., *et al.* "Solubility of nano-zinc oxide in environmentally and biologically important matrices." Environmental Toxicology and Chemistry, vol. 31, no. 1, 2012, pp. 93–99. https://doi.org/10.1002/etc.708.
25. Ng, C. T., *et al.* "Zinc oxide nanoparticles exhibit cytotoxicity and genotoxicity through oxidative stress responses in human lung fibroblasts and drosophila melanogaster." *International Journal of Nanomedicine,* Volume 12, 2017, pp. 1621–1637. https://doi.org/10.2147/IJN.S124403.
26. Nagajyothi, P. C., *et al.* "Antioxidant and anti-inflammatory activities of zinc oxide nanoparticles synthesized using Polygala tenuifolia root extract." *Journal of Photochemistry and Photobiology.* Vol 146, 2015, pp. 10–17. https://doi.org/10.1016/j.jphotobiol.2015.02.008.
27. Prasad, A. S. "Zinc is an antioxidant and anti-inflammatory agent: Its role in human health." *Frontiers in Nutrition,* vol. 1, 2014. https://doi.org/10.3389/fnut.2014.00014.
28. Datto, M. B., *et al.* "Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter." *The Journal of Biological Chemistry,* vol. 270, no. 48, 1995, pp. 28623–28628. https://doi.org/10.1074/jbc.270.48.28623.
29. Liu, J., *et al.* "The toxicology of ion-shedding zinc oxide nanoparticles." *Critical Reviews in Toxicology,* vol 46, no 4, 2016, pp. 348-384. https://doi.org/10.3109/10408444.2015.1137864.