Anti-Cancer and Stress Response Pathway Effects of Nanosilver and Sodium Ascorbate

by

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Abstract

Nanosilver (nAg) has superb antimicrobial, antiviral, antifungal, antiparasitic, and anticancer properties; and plays an important role in nanoscience, nanotechnology, and nanomedicine. Smaller nAg particles can enter cells and interact with the cellular components. The exposure dose, particle size, coating, and aggregation state, as well as the cell type or organism, are all large determining factors on the beneficial or toxic effects of nAg. Sodium ascorbate is a vital water-soluble antioxidant that can neutralize free radicals produced during illness. It has pro-oxidant effects in its oxidized form, and in this form is able to destroy cancer cells through the production of hydrogen peroxide. With the growing prevalence of cancer, there is an increasing need to find both new treatments and combinations of treatments to provide greater effectiveness.

Nanosilver was found to be more toxic to HCT116 human colon cancer cells (24-hour EC$_{50}$ of 78.43 ± 0.70 µg/mL) than to HIEC-6 human intestinal epithelial cells (no toxicity was observed for the treatment concentrations tested). Combined treatments of high dose sodium ascorbate with nAg demonstrated significantly increased toxicity to HCT116 cells as opposed to HIEC-6 cells, and increased the cell death from that observed with either of the treatments alone. The novel result was found that it is more toxic to cancer cells to use a combined treatment of high dose sodium ascorbate with low dose nAg; as opposed to low dose sodium ascorbate with high dose nAg, where a protective effect is seen. Nanosilver induced cell cycle arrest in the G2/M phase in HCT116 cells, and combined treatment with sodium ascorbate further increased this effect. This was found to be a nAg-specific effect and did not occur with silver nitrate. Cellular oxidative stress was not induced by nAg, however, mitochondrial oxidative stress was induced in HCT116 cells after 24 hours of treatment. No significant effect on mitochondrial
oxidative phosphorylation or the cellular ER stress response was found. Combined treatment of nAg with sodium ascorbate may be beneficial in decreasing WNT pathway signalling in cancer cells through the non-canonical pathway involving WNT5A.
Dedication

To my Lord and Saviour Jesus Christ, the Rest for my soul, and the Creator of this amazing and beautiful world – from the incredibly intricate workings of the cell to the vastness of the universe. “For from Him, and through Him, and to Him are all things. To Him be the glory forever.” (Romans 11:36)

Dedicated with much love to Mom and Dad, and to Elaine Bennett.
I am forever grateful for your love, guidance, and teaching; you are always in my heart.

Also in memory of my friend Majed Alshehri.

Until we meet again in heaven on that glorious day!
Acknowledgements

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Many thanks to Dr. Ashkan Golshani, Dr. David McMullin, Dr. Laurie Chan, and Dr. David Miller for kindly agreeing to serve as my thesis committee. You have all been instrumental during my academic studies, and I highly value your input and expertise. Thank you to Dr. David Miller for opening the world of toxicology to me. I was intrigued from the first lecture of your Principles of Toxicology course and chose to specialize in toxicology to learn more in this fascinating field.

Warm thanks to all the lab members past and present for your friendship and support – it has been wonderful to work with you all! Special thanks to Jessica Sheng, Rowida Mohamed, Haiyun Bo, Matt Clinch, Jacob Billingsley, Anand Chopra, Ramak Esfandi, Hemanta Adhikary, Stephanie Hewetson, Owen Hovey, Emily Brown, James Kennedy, Mary Daniel, and Magda Bugno. Jessica, it has been fantastic working so closely with you! Owen, your assistance and advice has been invaluable to me. Thank you for teaching me the techniques in our lab and for going above and beyond for me in so many ways. Rowida, thank you so much for all your support and for your help with the qRT-PCR analysis. Duale Ahmed, thank you so much for your assistance with the Seahorse experiments. Warm and grateful thanks to all the undergraduate students I have had the privilege of working with: Peter Stolarski, Julie Hamati, Tristen Bennett, and Oluwatomike Aribaloye. Special thanks to my friends Haiyun Bo, Rowida Mohamed, Kathleen Braden, Joel Bennett, Cindy Leung, Usman Khan, Houman Moteshareie, and Katrina van der Meer for always being there for me.
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Looking at plants and flowers and wondering how they function and grow lead me into studying the intriguing field of biochemistry in my undergraduate degree; and seeing the incredible beauty and complexity of God’s creation lead me into pursuing graduate studies in chemistry and toxicology. I am truly grateful to have this opportunity.
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List of Abbreviations

AgNO₃: Silver nitrate
AhR: Aryl hydrocarbon receptor
ALP: Alkaline phosphatase
AMPK: AMP-activated protein kinase
AP1: Activator protein 1
APC: Adenomatous polyposis coli
ARE: Antioxidant response element
ARNT: Ah receptor nuclear translocator
ATF-6: Activating transcription factor 6
ATP: Adenosine triphosphate
AVERT: Artificial Valve Endocarditis Reduction Trial
BCA: Bicinchoninic acid
BrdU: 5-Bromo-2’-deoxyuridine
BSA: Bovine serum albumin
BSO: Buthionine sulfoximine
cDNA: Complementary DNA
CAT: Catalase
CDC2: Cell division cycle protein 2 homolog
CDC25: Cyclin-dependent kinase 25
CDK1: Cyclin-dependent kinase 1
CKI: Cyclin kinase inhibitor
CMV: Cytomegalovirus
CNC: Cap’n’collar
CPRG: Chlorophenol red-β-D-galactopyranoside
Cul3: Cullin3
CYP: Cytochrome P450
DAVID: Database for Annotation, Visualization and Integrated Discovery
DHA: Dehydroascorbate
DLA: Dalton’s lymphoma ascites
DMEM: Dulbecco's modified eagle medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DRE: Dioxin response element
DTT: Dithiothreitol
EC₅₀: Half maximal effective concentration
ECAR: Extracellular acidification rate
EDTA: Ethylenediaminetetraacetic acid
EpRE: Electrophile response element
ER: Endoplasmic reticulum
FBS: Fetal bovine serum
FCCP: Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FSC-A: Forward scatter area
FSC-H: Forward scatter height
GLUT: Glucose transporters
GSH: Glutathione
HCT116: Human colon carcinoma cells
HEK293T: Human embryonic kidney cells, transformed
HEPES: 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESC: Human embryonic stem cell
HGF: Human gingival fibroblasts
HIEC-6: Human intestinal epithelial cell 6
HIF-1α: Hypoxia-inducible factor-1α
HIV: Human immunodeficiency virus
HO-1: Heme oxygenase 1
H₂O₂: Hydrogen peroxide
ICP-MS: Inductively Coupled Plasma Mass Spectrometry
IFN-γ: Interferon-γ
IL: Interleukin
IRE-1α: Inositol-requiring transmembrane kinase/endoribonuclease 1α
KCl: Potassium chloride
Keap1: Kelch-like ECH-associated protein 1
KEGG: Kyoto Encyclopedia of Genes and Genomes
KH₂PO₄: Potassium dihydrogen phosphate
LC₅₀: Lethal concentration, 50%
LDH: Lactate dehydrogenase
MAPK: Mitogen-activated protein kinase
MgCl₂: Magnesium chloride
MgSO₄: Magnesium sulfate
MLKL: Mixed lineage kinase domain-like pseudokinase

MMP: Matrix metalloproteinase

mTOR: AMP-activated protein kinase (AMPK)/mammalian target of rapamycin

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NAC: N-acetyl-L-cysteine

NaCl: Sodium chloride

nAg: Nanosilver

Na₂HPO₄: disodium hydrogen phosphate

NCS: Newborn calf serum

NF-κB: Nuclear factor kappa B

NP-40: Nonidet P40 Substitute

Nrf1: Nuclear factor erythroid 2-related factor 1

Nrf2: Nuclear factor erythroid 2-related factor 2

Nrf3: Nuclear factor erythroid 2-related factor 3

OCAR: Oxygen consumption rate

PAK: Serine/threonine protein kinase

PANC-1: Human pancreas ductal adenocarcinoma cell

PBS: Phosphate-buffered saline

PC-12: Rat pheochromocytoma cells

PCNA: Proliferating cell nuclear antigen

PEI: Polyethylenimine

PERK: Protein kinase RNA-like endoplasmic reticulum kinase

PHD: Prolyl hydroxylase
PI: Propidium iodide
PMSF: Phenylmethylsulfonyl fluoride
PORCN: Porcupine
PP2A: Protein phosphatase 2A
PVDF: Polyvinylidene difluoride
PVP: Polyvinylpyrrolidone
qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction
RAW264.7: Mouse macrophage cells
RIP1: Receptor-interacting serine/threonine-protein kinase 1
RIP3: Receptor-interacting serine/threonine-protein kinase 3
RNA: Ribonucleic acid
ROS: Reactive oxygen species
SA: Sodium ascorbate
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDS: sodium dodecyl sulfate
S.E.M: Standard Error of the Mean
SFN: Sulforaphane
SGOT: Glutamate oxalate transaminase
SGPT: Glutamate pyruvate transaminase
SH-SY5Y: Human neuroblastoma cells
SOD: Superoxide dismutase
SSC-A: Side scatter area
STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

SUMO: Small ubiquitin-related modifier

SVCT: Sodium-coupled vitamin C transporter

TBHQ: Tert-butylhydroquinone

TCDD: 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin

TCE: Trichloroethanol

TCF: T-cell factor

TEM: Transmission Electron Microscopy

TGF-β: Transforming growth factor-β

TNF-α: Tumour necrosis factor-α

TRIM28: Tripartite motif containing 28

UV-Vis spectrum: Ultraviolet/visible spectrum

WNT: Wingless/Integrated-1
Chapter 1: Introduction

1.1 Nanosilver Background and Applications

Nanosilver (nAg) consists of small silver particles that are 1 – 100 nm in at least one dimension and can be formed in various shapes. Nanosilver is also commonly referred to as silver nanoparticles, colloidal silver, or millimicron silver. Silver has long been used medicinally for thousands of years to purify water and to treat wounds and infections, and was used extensively for a myriad of medical conditions in Europe and America from around 1900 until the early 1940s with the isolation and subsequent widespread use of antibiotics (Dean et al., 2001; Knuckles et al., 2012). Citrate-stabilized colloidal silver was first synthesized by M. Carey Lea in 1889, with his method producing 7 – 9 nm sized particles (Frens & Overbeek, 1969; Lea, 1889; P. Wang et al., 2018).

Nanosilver has superb antimicrobial, antiviral, antifungal (Prateeksha et al., 2019), antiparasitic (Saini et al., 2016; S. K. Singh et al., 2012), and anticancer properties; and has even been reported to have potential anti-aging effects (Radwan et al., 2020). Nanosilver binds to electron donors in proteins such as sulfur, oxygen, phosphorus, and nitrogen (Mikhailova, 2020); and has been found to block viral binding and entry into cells and to inhibit viral replication by binding to the viral surface proteins (such as the glycoprotein knobs on the surface of human immunodeficiency virus [HIV]), and disrupting their structure and function (Elechiguerra et al., 2005; Hu et al., 2014; Sun et al., 2005; Trefry & Wooley, 2013). Nanosilver is effective against a wide range of viruses including HIV, herpes, and influenza; and has recently been proposed as a potential treatment for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) due to its ability to bind to the spike protein and effectively block the replication of SARS-CoV-2 in...
infected cells *in vitro* (Hati & Bhattacharyya, 2020; Jeremiah et al., 2020; Pilaquinga et al., 2021).

Nanosilver is highly effective against both gram positive and gram negative bacteria (Kalińska et al., 2019; Lu et al., 2013; Mikhailova, 2020; Zhang et al., 2015) as well as against multidrug-resistant bacteria (Barros et al., 2018; Xueqing Chen et al., 2019; dos Santos et al., 2021; Halawani et al., 2020; Liao et al., 2019). Encouragingly, successive exposure of the multidrug-resistant bacteria to nAg has not been found to result in decreased antimicrobial effectiveness or bacterial tolerance (dos Santos et al., 2021).

The antimicrobial effects of nAg are due to many factors including the release of silver ions, the induction of oxidative stress and damage (Liao et al., 2019), disruption of the bacterial cell wall resulting in increased permeability, binding to the thiol groups of bacterial proteins inhibiting their function (Eryılmaz et al., 2018), and the upregulation of bacterial pro-apoptotic genes and the downregulation of anti-apoptotic genes (Halawani et al., 2020).

Nanosilver is increasingly being used in many commercial, medical, and household applications such as in bandages, medical device coatings, electronics, textiles, sport and military clothing, coatings on appliances and counters, cosmetics, and food packaging (León-Silva et al., 2016; Panyala et al., 2008). Thus, human exposure to nAg is steadily increasing with much remaining to be discovered about the interactions of nAg in the human body and its effects both alone and in combination with other compounds.

Human use of nAg leads to its release into the environment in wastewater and sewage sludge, which may be used for irrigation or as fertilizer, thus effecting the agricultural soils, soil bacteria, and earthworms (Jia Liu et al., 2018). Nanosilver released into the environment does not stay in a free particulate form, instead mainly forming insoluble silver sulfide compounds.
with only minor amounts being released as free toxic silver ions, greatly reducing the potential toxicity of nAg in the environment (Baccaro et al., 2018; Blaser et al., 2008; Kaegi et al., 2011; Jia Liu et al., 2018; Massarsky et al., 2014).

1.2 Nanosilver Adsorption, Distribution, Metabolism, and Excretion

Depending on work, lifestyle, and medical treatments, human exposure to nAg can occur through various avenues including inhalation (such as during manufacturing or with the use of sprays), ingestion (such as from food packaging or if taken medicinally), through skin contact (with products containing nAg such as cosmetics, bandages, or treated fabrics), or through other means such as with the use of coated medical catheters, bone cement and implants, coated instruments, coated contact lenses, or certain eye drops (Chaloupka et al., 2010; Wen et al., 2016).

The exposure route determines which organs and tissues are the most exposed. Exposure through inhalation targets the nasal cavities and lungs, intravenous administration results in accumulation in the liver and spleen, and oral administration leads to initial accumulation in the stomach and gastrointestinal tract and subsequent distribution to the organs (Wen et al., 2016; Xue et al., 2012). Intraperitoneal injection of mice lead to accumulation mainly in the liver, spleen, and lungs, with lower levels in the kidneys, heart, and brain (Skomorokhova et al., 2020). Rats and mice orally treated by gavage with nAg for 28 days had the highest accumulation in the liver and spleen, with lower levels in the other organs (Gan et al., 2020; Van Der Zande et al., 2012). When Sprague Dawley rats were treated with nAg by oral gavage for 13 weeks, no adverse effects on their body weight, food intake, hematology, or reproductive systems were found. However, dose-dependent silver accumulation occurred in various tissues (particularly in
the kidney, liver, jejunum, and colon) and was significantly higher in the female rats than the male rats (Boudreau et al., 2016). A sex-related difference in silver accumulation was also found in a different study where twice as much silver was found to accumulate in the kidneys of female rats compared to male rats after oral treatment with nAg for 90 days (Kim et al., 2009; Kim et al., 2008, 2010). In the liver tissue, the nAg is mainly localized near the blood vessels, and is found within endosomes and lysosomes in the Kupffer cells (Lee et al., 2013).

Nanosilver can cross the blood-brain, blood-milk, and placental barriers (Melnik et al., 2013; Oh et al., 2016; Tang et al., 2008; Wang, Qu, et al., 2013), and inhalation of nAg can lead to exposure of the brain through the olfactory bulb (Haase et al., 2012). Nanosilver has been found to mainly be excreted by the liver in the bile with only trace amounts being excreted in the urine (Gan et al., 2020; Skomorokhova et al., 2020; Van Der Zande et al., 2012).

1.3 Effect of Physical Characteristics and Coatings

The physical characteristics of the nAg including size, shape, surface coating, surface charge, protein corona formation, and aggregation state all effect the exposure level and biological responses to the nAg. Larger nAg particles may interact with the cell membrane and surface receptors such as serine/threonine protein kinase (PAK), mitogen-activated protein kinase (MAPK), and protein phosphatase 2A (PP2A) (Verano-Braga et al., 2014); while smaller nAg particles that are on the same scale as deoxyribonucleic acid (DNA) and proteins may enter the cells via endocytosis or cause lipid peroxidation and damage and diffuse across the lipid membrane where they can and interact with biological molecules within the cell (see Figure 1) (Chaloupka et al., 2010). In fact, nAg and silver ions have been found to bind to various sulfur containing proteins and peptides involved in the cellular antioxidant response including
glutathione, glutathione S-transferase, peroxiredoxin, thioredoxin, thioredoxin peroxidase, and superoxide dismutase; as well as to other proteins such as myosin, 60S ribosomal protein and 40S ribosomal protein (Mao et al., 2016a; Verano-Braga et al., 2014; Xu et al., 2019; Zhang et al., 2014). During endocytosis, nAg particles are incorporated into early endosomes formed from the cell membrane, which then develop into late endosomes followed by lysosomes, which have a lower internal pH resulting in increased release of silver ions (Greulich et al., 2011; Mao et al., 2016a). Nanosilver has also been postulated to be taken into the cell through copper transport channels (Armstrong et al., 2013; Havelaar et al., 1998; Mao et al., 2016a). The function and type of cell greatly effects the amount of nAg that is taken in, with cells such as phagocytes taking in nAg independent of size or surface coating (Chorley et al., 2014).

Smaller nAg particles release higher amounts of silver ions through surface oxidation, generally resulting in greater biological effects and antibacterial activity than larger particles (Karlsson et al., 2014; Skomorokhova et al., 2020). Aggregation of the nAg can sometimes occur in cell culture media depending on the stability of the nAg, the surface coating used, and the handling method (Huang et al., 2015).

Polyvinylpyrrolidone (PVP) and citrate are common nAg coatings used to stabilize the nanoparticles and prevent aggregation, and on their own they have not been found to cause the biological effects that are observed with the coated nAg (Armstrong et al., 2013; Das et al., 2014; Lin et al., 2018; Wu et al., 2015, 2016). Other nAg surface coatings include polyethylene glycol, polyvinyl alcohol, cellulose, and chitosan (Satapathy et al., 2013).

It is worth noting that the dosage of the nAg also plays a role in determining the cellular effects and toxicity. Lower sub-toxic doses are more relevant to the actual environmental exposure levels than does that are a high and toxic concentration, however, many of the studies
used treatment dosages at the half maximal effective concentration (EC\textsubscript{50}) level (Armstrong et al., 2013).

Silver ion controls are important in studies involving nAg in order to control for the effects due to released silver ions as opposed to nanoparticle-specific effects. However, the Ag\textsuperscript{+} ion control cannot be used at the same concentration as the nAg treatment dose otherwise the AgNO\textsubscript{3} will be much more toxic due to the fact that there are more silver ions present than are in the nAg solution (Armstrong et al., 2013; Zielinska et al., 2018). A relevant silver ion control treatment level is generally determined using inductively coupled plasma mass spectrometry (ICP-MS) to measure the precise concentration of silver ions released from the nAg solution (Lin et al., 2014; Manshian et al., 2015; Z. Wang, Liu, et al., 2013; Zielinska et al., 2018). An alternative way of determining a relevant silver ion control concentration is through determining the concentration that gives the same cell viability as the nAg (Garcia-Reyero et al., 2014). Alternatively, the nAg can be removed by centrifugation from the cell culture media, and the cells then treated with this media, which would contain any released silver ions from the nAg (Srikar et al., 2016; Verano-Braga et al., 2014). Nanoparticle controls are also occasionally used such as cerium or polystyrene nanoparticles (Kawata et al., 2009; Lin et al., 2014; Prasad et al., 2013a).
Figure 1: Effects of nanosilver on the cellular stress response pathways. Smaller sized nanosilver (~10 nm diameter) enters the cell either through being taken up into endosomes/lysosomes by endocytosis or through simple diffusion across the cell membrane (potentially due to induced lipid peroxidation and disruption of the plasma membrane). Larger sized nanosilver or large aggregates of nanosilver cannot enter the cell by these means, but can instead activate various receptor-mediated signalling mechanisms, such as through PAK, MAPK, and PP2A. Increased lipid peroxidation causes increased lactate dehydrogenase (LDH) release from the cell due to cell membrane damage. Nanosilver treatment results in an increase in reactive oxygen species (ROS), and the extrinsic apoptotic pathway may be induced. The levels of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) are affected and an increase in oxidative stress response gene expression occurs. In the nucleus, an increase may occur in genotoxicity (DNA damage, DNA base oxidation, DNA adducts, DNA strand breaks, and chromosomal aberrations) and epigenetic changes (DNA methylation, various histone tail modifications, and changes in non-coding RNA expression), potentially in a transient manner. Mitochondrial dysfunction, decreased mitochondrial membrane potential, decreased ATP production, and mitochondrial-mediated intrinsic apoptosis may also occur. As well, nanosilver treatment increases the protein and gene expression levels of p53, leading to anti-cancer effects. High dose nanosilver treatment disrupts endoplasmic reticulum (ER) homeostasis and induces the ER stress response through activated protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF-6), and inositol-requiring transmembrane kinase/endoribonuclease 1α (IRE-1α), and their respective pathways. Contact between the ER and the mitochondria increases with nanosilver treatment, and increased transfer of calcium from the ER to the mitochondria occurs, resulting in increased calcium levels in the mitochondria. (Cameron et al., 2018)
1.4 Human Clinical and Case Studies with Nanosilver

Although silver in various forms (silver salts, silver compounds, colloidal silver, or silver combined with protein) has long been used medicinally (Dean et al., 2001), there are currently only a few clinical and case studies reporting the medicinal use of nAg in human treatment. The most recent case study is by Singh et al (2018), and reports the dramatic and complete resolution of metastatic head and neck squamous cell cancer in a 78-year old male who had been repeatedly and aggressively treated with surgery, radiation, and chemotherapy. These conventional treatments ultimately failed, and the patient developed metastases to his lymph, heart, liver, and lung, as well as recurrences in the original sites. While on hospice care and with no further conventional treatment options available, the patient began making a 0.09 – 0.15 ppm nAg solution containing 3 – 12 nm particles, straining it to remove any silver precipitate, and drinking 120 mL a day. This treatment resulted in a basal blood silver ion concentration of 32 ng/g which rose to 46 ng/g one hour after drinking 60 ml nAg. No intact nAg particles were found in his blood serum or excreted in his urine. Remarkably, over the next three months of self-treatment with nAg his cancer completely resolved, and he has remained cancer free 36 months later as of the writing of the case study and continues to take a daily maintenance dose. His ingestion of nAg was safe and had no adverse effects or toxicity, no myelosuppression, no liver or kidney issues, and no skin discoloration while displaying remarkable anti-cancer activity (Singh et al., 2018).

A single-blind, controlled, cross-over, intent-to-treat clinical study was performed with 60 healthy patients treated orally with 10 ppm (5 – 10 nm) or 32 ppm (25 – 40 nm) solutions of commercially obtained nAg; with no toxicity or changes in metabolic, hematologic, urine, or physical characteristics found (Munger et al., 2014). Additionally, no effects on platelet
aggregation was found in a placebo-controlled, single-blind, dose-monitored, cross-over study of 18 healthy humans taking oral nAg for 2 weeks (Smock et al., 2014).

Three patients with HIV in 1997 were treated with a combination of both oral and intravenous mild silver protein, which is a solution containing silver combined with protein. They all had rapidly deteriorating conditions and were experiencing extremely low energy levels and weakness that prevented them from working or even walking in one case. They were initially treated orally with increasing concentrations from 40 ppm to 400 ppm over a one-month period, at which point intravenous treatment was added with increasing concentrations of 40 ppm, 400 ppm, and finally 1500 ppm in a saline solution which contained dimethyl sulfoxide (DMSO). The patients all experienced Jarisch-Herxheimer-like reactions of nausea, vomiting, fever, headache, and muscle pain after the infusions, with these symptoms decreasing with additional treatments. One patient experienced pancytopenia at the highest dose that resolved when the dosage increase was made more gradual. It was concluded that the intravenous mild silver protein treatment was a safe and effective anti-viral treatment at 1 – 2 infusions per week at the 400 ppm dose. The patients were closely monitored during this treatment and all experienced dramatically decreased viral loads and restored energy levels; with the beneficial effect being to such an extent that they went from demanding the treatment to declining it since they no longer felt ill (Dean et al., 2001).

Regarding nAg use in medical devices, nAg is beneficial when incorporated into bandages and greatly decreases wound healing time as well as preventing infection with no adverse effects (Lackner et al., 2008). Beneficial anti-microbial effects have also been found in a randomized clinical trial with 66 patients given removable acrylic retainers containing 40 nm nAg at a concentration of 500 ppm. After 7 weeks of use, the patients that received the nAg
containing retainers had significantly reduced levels of *Streptococcus mutans* infection (Farhadian et al., 2016). As well, the use of silver-impregnated external ventricular drainage catheters has successfully prevented the occurrence of catheter-associated ventriculitis in 19 patients with acute occlusive hydrocephalus with no reported toxic side effects. This was a significant decrease in infection with 5 of the 20 patients in the control group contracting catheter-associated ventriculitis (Lackner et al., 2008). However, care must be taken on the location and desired function of medical devices with silver coatings. For example, the Artificial Valve Endocarditis Reduction Trial (AVERT) in 1998 that used a prosthetic silicone heart valve coated with elemental silver was halted after 2 years due to paravalvular leakage, since this was thought to be caused by inhibited fibroblast function and decreased growth into the sewing cuff of the valve (Grunkemeier et al., 2006; Jamieson et al., 2009).

It is important to note that chronic exposure to improperly made and sized nAg may result in argyria, a condition where silver is deposited in the skin microvessels, intestines, and glomeruli. Upon contact with light on the skin, the silver undergoes photoreduction and subsequently binds to sulfur or selenium forming particulate that results in a permanent bluish-grey discolouration of the skin (Hadrup & Lam, 2014; Jingyu Liu et al., 2012). Argyrosis occurs when silver is deposited in the eyes (Wen et al., 2016). However, these conditions only occur when the nAg is made incorrectly with silver ions produced or if the silver particles are not actually in the nm range and are thus not small enough to be efficiently excreted by the body. Manufacturing technology has improved greatly in recent years, and no cases of argyria have resulted from modern day correctly manufactured nAg (Frazer, 2012).
1.5 Nanosilver, Inflammation, and Wound Healing

Inflammation occurs as the result of an immune response to injury involving leucocyte cells which infiltrate the area, release cytokines such as interleukin-6 (IL-6), IL-1β, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and transforming growth factor-β (TGF-β) to stimulate the production of acute phase plasma proteins among other biochemical and physiological changes to facilitate healing. Acute inflammation for a short time frame helps the healing process; however, chronic inflammation is very detrimental and may lead to damage and diseases such as arthritis and cancer (Franková et al., 2016; Gabay, 2006; Rakoff-Nahoum, 2006).

Wound healing involves a process of inflammation, proliferation, and tissue remodelling (Franková et al., 2016). Nanosilver treatment induces inflammation both in vitro (Chorley et al., 2014; Gallorini et al., 2016; Hackenberg et al., 2011; Manshian et al., 2017; Mishra et al., 2016; Prasad et al., 2013a) and in vivo (Lee et al., 2013; Manshian et al., 2017; Park et al., 2010); however, initial short-term increase in inflammation leads to faster wound healing with nAg treatment (Franková et al., 2016; Orlowski et al., 2018; Tian et al., 2007).

1.6 Effects of Nanosilver on the Gastrointestinal Tract

Since nAg is an effective antimicrobial agent, and human intestinal microbiota may be sensitive to nAg (Das et al., 2014), care must be taken with the duration and use of nAg in human treatment to mitigate potential detrimental effects. Human intestinal microbiota consist mainly of bacteria in the phyla Bacteroidetes (Gram negative) and Firmicutes (mainly Gram positive), with some from Proteobacteria and Actinobacteria. Nanosilver treatment of the cultured synthetic human stool mixture RePOOPulate decreased bacterial respiration levels, and
shifted the distribution of the bacterial microbes by decreasing the levels of Gram negative *Bacteroides ovatus*, Gram positive *Roseburia faecalis*, Gram positive *Eubacterium rectale*, Gram positive *Roseburia intestinalis*, and Gram positive *Ruminococcus torques*; while increasing the levels of Gram negative *Raoultella sp.* and *Escherichia coli* (Das et al., 2014). In general, Gram positive bacteria (with the thick peptidoglycan layer) appear to be less effected by nAg than Gram negative bacteria (Kalińska et al., 2019; Mikhailova, 2020). Nanosilver treatment of fermentation cultures of human fecal matter did not affect the core bacterial community, although an increase in the relative abundance of *Firmicutes* and a decrease in *Bacteroidetes* was found. Co-treatment with the probiotic *Bacillus subtilis* prevented nAg-induced effects on bacterial cell motility, translation, and metabolic pathways (Cattò et al., 2019). In the fruit fly microbiome, nAg increased the levels of Gram positive *Lactobacillus*, while decreasing the levels of Gram negative *Sphingdomomas* and *Rhizobiales* (Han et al., 2014). Interestingly, in obese mice, oral nAg treatment was beneficial and mitigated obesity-induced inflammation, increased gut microbial diversity, and resolved gut microbial dysbiosis (Jia et al., 2021).

Not much is known about the potential effect of nAg on intestinal permeability. Increased permeability accross the gastrointestinal tract due to damage or inflammation may result in leaky gut syndrome, allowing molecules to cross the intestinal epithelium that should be excluded. Oral nAg treatment of male and female rats for 13-weeks has been found to alter the expression of genes involved in various types of cell junctions including tight junctions, focal adhesions, adherens junction, and hemidesmosomes. A sex-specific result was found where the cell junction genes were upregulated in the ileum of female rats, while being either downregulated or having no change in the male rats (Orr et al., 2019).
1.7 Nanosilver and Cancer

Cancer forms due to a combination of environmental, physiological, and genetic factors (Buttacavoli et al., 2018), and cancer cells have altered metabolism, energy production processes, and gene expression (Gandhi et al., 2021). Novel treatments and combinations of treatments are of great interest in treating cancer due to the limited effectiveness of many current cancer treatments and the development of multi-drug resistant cancer cells rendering the treatments ineffective (Kovács et al., 2016; Yesilot & Aydin, 2019), as well as the extremely limited options to improve the quality of life for patients with late-stage cancers (Ichim et al., 2011).

Nanosilver treatment of cancer has had promising results in both cell culture and animal studies, as well as in a recent human case study where nAg treatment resulted in spontaneous remission in the patient (Singh et al., 2018). Nanosilver treatment also successfully increases the lethality of radiation (Habiba et al., 2019; Liu et al., 2016; Wu et al., 2015; Zheng et al., 2013) and various other drugs used in the treatment of cancer such as the chemotherapy agent Cisplatin (Gurunathan et al., 2015; Yuan & Gurunathan, 2017), the monocarboxylic ionophore, Salinomycin, which is used to target cancer stem cells (Zhang & Gurunathan, 2016), and the autophagy inhibitor Wortmannin (Lin et al., 2014). Nanosilver has also been found to be effective against multi-drug resistant cancer cells by inhibiting their efflux ability through ABC transporters and thus increasing intracellular drug accumulation, as well as triggering apoptosis (Kovács et al., 2016). Treatment of multi-drug resistant colon adenocarcinoma cancer cells with nAg in combination with the chemotherapy drugs Methotrexate, Cisplatin, Carmustine, Bleomycin, Vinblastine, and Verapamil showed increased lethality than with either the nAg or the drug treatment alone (Kovács et al., 2016).
Anti-cancer mechanisms induced by nAg include induction of apoptosis, inhibition of cell migration, inhibition of cell proliferation, induction of oxidative stress, DNA damage, mitochondrial damage, and membrane damage and leakage (Ferreira et al., 2020). Programed cell death through necroptosis has also been observed in human pancreas ductal adenocarcinoma (PANC-1) cells with increased expression of receptor-interacting serine/threonine-protein kinase 1 (RIP1), receptor-interacting serine/threonine-protein kinase 3 (RIP3), and mixed lineage kinase domain-like pseudokinase (MLKL) (Zielinska et al., 2018). Levels of the pro-apoptotic protein Bax increased, the anti-apoptotic protein Bcl-2 decreased, and levels of the tumor suppressor transcription factor, p53, also increased (Zielinska et al., 2018). This transcription factor is involved in inducing cell cycle arrest, apoptosis, and cell senescence (Zhang & Gurunathan, 2016). Cell cycle arrest prevents the replication of damaged DNA and allows for DNA repair to occur, however if the stress and damage is too great apoptosis is triggered (Blanco et al., 2018; Sahu et al., 2015). Interestingly, p53 signalling has been found to be essential for the nAg induced MAPK signalling leading to mitochondrial mediated apoptosis in HCT116 cells, which did not occur with p53 knocked out of the cells (Satapathy et al., 2015).

1.7.1 Nanosilver and Colon Cancer

Worldwide, cancer is the foremost cause of death with one in every six deaths being due to cancer; and lung, colon, breast, and prostate cancer are the most commonly occurring cancers in 2020 (Henley et al., 2020). Colon cancer is the second most common cancer and cause of cancer death worldwide (Wen et al., 2019). Early-stage colon cancer is treated with tumor resection and chemotherapy. However, half of colon cancer patients develop drug resistance and metastasis. A quarter of colon cancer patients have liver metastasis at the time of initial
diagnosis, and half of the patients develop metastasis in the liver within three years of their first surgery. Advanced colon cancer is difficult to treat successfully with conventional treatments due to developed drug resistance and the occurrence of metastasis (Chakrabarti et al., 2020; Wen et al., 2019), and 5-fluorouracil is the only chemotherapy drug available for treating advanced colon cancer (Gurunathan et al., 2018).

Nanosilver treatment of HCT116 colon cancer cells demonstrated increased cell death which was not seen in comparative studies with normal human colon epithelial (FHM) cells, and increased expression of the proapoptotic markers p53, p21, and Bax (Acharya et al., 2021; Satapathy et al., 2013); as well as inducing cell-cycle arrest, mitochondrial dysfunction, and DNA damage (Gurunathan et al., 2018). The protein p21 is a cyclin kinase inhibitor (CKI) involved in cell cycle arrest downstream of p53 (Gartel & Tyner, 1999). Nanosilver also induced oxidative stress and disrupted the cytoskeleton and cell membrane in HCT116 colon cancer cells (Xiao et al., 2019). Additionally, treatment of Caco-2 human colon cancer cells resulted in significant cell death with low levels of nAg treatment (Zein et al., 2020).

High oxidative stress was caused by nAg treatment in LoVo human colon cancer cells, and analysis using gene ontology and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database indicated that proteins involved in the proteasome, translation initiation, oxidative stress response, and cell death were upregulated; while proteins involved in cell growth, the mitochondrial electron transport chain, spliceosome function, and mitochondrial translation all decreased. Although the protein targets are unknown, nAg treatment activated the post-translation modification small ubiquitin-related modifier (SUMO) pathway as seen with the upregulation of SUMO2, a member of the SUMO protein family, and tripartite
motif containing 28 (TRIM28), which is an E3 ligase involved in SUMOylation (Verano-Braga et al., 2014).

1.7.2 Nanosilver and Breast Cancer

Breast cancer accounts for 15% of all cancer deaths and effected approximately two million women in 2018. Treatment includes mastectomy or lumpectomy surgery, radiation, chemotherapy, and hormonal therapy treatment (Gandhi et al., 2021). Nanosilver treatment has been found to be selectively toxic to MCF-7 breast cancer cells, as compared to NIH3T3 normal fibroblast cells, and to trigger apoptosis through downregulation of proliferating cell nuclear antigen (PCNA) and Cyclin-D1 which are involved in cell cycle regulation and cell proliferation (Gandhi et al., 2021; Khateef et al., 2019).

Anti-cancer activity of nAg has been demonstrated with multidrug-resistant MCF-7/ADR cells and multidrug-resistant B16 melanoma cells as compared to treatment of the corresponding non-resistant HeLa and MCF-7 cancer cells. Increased effectiveness was found when using nAg modified with the cell penetrating TAT peptide. This was also seen in vivo, with female nude mice with multidrug resistance B16 melanoma cells simulating late-stage multidrug resistant cancer. Nanosilver treatment inhibited growth of the tumors, and the TAT-modified nAg was found to be as effective as treatment with doxorubicin with more than 85% growth inhibition but without the accompanying debilitating doxorubicin adverse effects (Liu et al., 2012). Biosynthesized nAg was found to be cytotoxic to human breast cancer cells SKBR3 and 8701-BC, and human colon cancer cells HT-29, HCT 116, and Caco-2; with much lower toxicity found in non-cancerous HB2 mammary epithelial cells and the nAg treatment having a selectivity index similar to the commonly used chemotherapy drug doxorubicin. Out of all the
cells tested, the SKBR3 breast cancer cells were the most susceptible to the nAg, and had decreased cell motility, decreased colony forming capacity, induced apoptosis, and increased autophagy. Matrix metalloproteinases (MMPs) are in integral to cell motility, and it was found that nAg treatment decreased the levels of MMP-2 and MMP-9. Protein-protein interaction analysis using STRING and bioinformatics pathway analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID), indicated that nAg treatment of the SKBR3 breast cancer cells affected the expression of proteins involved in pathways in the mitochondria, ER, oxidative stress response, apoptosis regulation, and downregulated enzymes involved in glycolysis (Buttacavoli et al., 2018).

1.7.3 Nanosilver and Lung Cancer

Lung cancer is the most common cause of all cancer deaths reported in the United States (Ardila et al., 2019). In an animal study with female mice having tumors made from KLN 205 murine lung squamous tumor cells, one-time peritumoral injection of 5 nm nAg decreased the tumor size in both the immune competent and immune deficient mice. In the immune deficient mice, the tumor growth rate returned to its pre-treatment state, while interestingly in the immune competent mice the tumoral growth rate was greatly decreased and never recovered, potentially indicating that the nAg treatment triggered an immune response in the mice enabling them to control the tumor growth (Manshian et al., 2017).

1.7.4 Nanosilver and Prostate Cancer

Prostate cancer was the second most common cancer in men and the fifth most common cause of death in 2012 (He et al., 2016). Treatment of prostate cancer PC-3 cells resulted in cell
death, decreased levels of stat 3, Bcl-2, and survivin; and triggered extrinsic apoptosis through increased caspase-3 (He et al., 2016). The Bcl-2 protein family is involved in mitochondrial mediated apoptosis; with Bcl-2 having anti-apoptotic effects while Bax and Bak are pro-apoptotic proteins (Zhang & Gurunathan, 2016).

Nanosilver regulates autophagy in PC-3 cells by causing lysosomal membrane damage, inhibiting lysosomal protease activity, and overall decreasing the number of functioning lysosomes. This in turn inhibits autophagy, which the cellular-mediated degradation of proteins, sugars, lipids, and some organelles via the lysosome (Tanida, 2011) and is an important mechanism in the survival and grown of cancer cells (Chen et al., 2020). Additionally, nAg induced a hypoxic response and depleted cellular energy levels, stimulating the activation of autophagy through AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling (Chen et al., 2020).

1.7.5 Nanosilver and Bladder Cancer

Bladder cancer is the fifth most common cancer in the United States and the eleventh most common cancer world-wide. In the United States alone, this results in 17,000 deaths per year, and has less than 15% survival rate of more than 2 years if left untreated (Ferreira et al., 2020). Seventy percent of bladder cancer is superficial and non-muscle invasive when initially diagnosed, however, the bladder cancer will reoccur in half of the patients within 4 years and a tenth of the patients with now have progressed to the more serious muscle-invasive bladder cancer. Treatment for bladder cancer includes transurethral resection of the tumor and immunotherapy, however, there are limited treatment options making the discovery of novel treatment options a necessity. Nanosilver inhibits the cell proliferation and migration of bladder
cancer 5637 cells and induces apoptosis. Impressively, intravenous nAg treatment of female mice with induced non-muscle invasive bladder cancer resulted in 57.13% tumor regression, with 14.28% of them now having normal urothelium and the remaining 42.85% only having flat hyperplasia which is viewed as a benign lesion (Ferreira et al., 2020).

1.7.6  **Nanosilver and Lymphoma**

Mice with tumors formed using Dalton’s lymphoma ascites (DLA) were treated with nAg via intraperitoneal injection for 15 days at a dose of 500 nM; with this treatment level being the EC$_{50}$ for DLA cells and resulting in activated caspase-3, induced DNA fragmentation, and apoptosis in the DLA cells. Surprisingly, no toxicity or adverse effects such as appetite loss, reduction in body weight, fatigue, or change in fur color change were found in the non-cancerous control mice treated with 500 nM nAg; and the tumor-bearing mice greatly improved with the treatment. The number of malignant DLA cells in their peritoneal fluid significantly decreased, the excess ascetic fluid decreased by 65%, they regained normal weight, white blood cell, and platelet count, and they lived 50% longer than the untreated tumor-bearing mice (Sriram et al., 2010). Interestingly, intraperitoneal nAg treatment for 10 days of mice with tumors formed using DLA cells was as effective as treatment with the chemotherapy drug 5-Fluorouracil (Jacob & Shanmugam, 2015). The nAg treated mice had decreased tumor volume and lived 46.52% longer than the control mice that had not been treatment. Nanosilver treatment successfully prevented the increased angiogenesis, hypercalcemia, lipid peroxidation, cell damage and membrane leakage as indicated by lactate dehydrogenase (LDH) levels, and prevented the decreased levels of the antioxidant proteins superoxide dismutase (SOD) and glutathione (GSH) that were observed in the non-treated mice. Liver damage indicated by leakage of the liver enzymes
glutamate oxalate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP) into the blood serum was also prevented with the nAg treatment. It was found that the nAg induced both intrinsic and extrinsic apoptosis in the tumor cells with increased gene expression of p53, cytochrome C, caspase-3, caspase-8, caspase-9, and caspase-12 (Jacob & Shanmugam, 2015).

1.8 Ascorbic Acid and Cancer

Vitamin C (L-ascorbic acid, ascorbate) is a vital water-soluble antioxidant that can neutralize the free radicals and reactive oxygen species (ROS) that are produced during sickness. Vitamin C is made by most animals from glucose but not by humans, and thus must be obtained through the diet (Carpenter, 2012; Ngo et al., 2019). Humans, primates, guinea pigs, and fruit bats all have inactivating mutations in the gene for gulonolactone oxidase, and thus lack the last enzyme in the pathway to synthesize vitamin C (Duarte & Lunec, 2005). Vitamin C is essential as an antioxidant, helps to recycle oxidized antioxidants such as alpha-tocopherol and glutathione, and functions as a cofactor in many biological processes such as in the formation of collagen, carnitine, catecholamines, vasopressin, and cortisol (Boyera et al., 1998; Khoshnam-Rad & Khalili, 2019). Scurvy is caused by vitamin C deficiency, manifesting in physical deterioration, weakness, loose gums, fragile skin, bleeding, and inhibited wound healing due to the lack of collagen production (Boyera et al., 1998). This lack of collagen production is due to the fact that vitamin C is required by the prolyl hydroxylase (PHD) enzyme to hydroxylate collagen and cause it to become the mature collagen protein.

Ascorbate is an excellent reducing agent, and readily donates electrons through two consecutive one-electron oxidation reactions, converting through an ascorbate free radical from
into dehydroascorbate (DHA) (see Figure 2). Dehydroascorbate can be reduced back to ascorbate by glutathione or thioredoxin reductase; or if it is not reduced, DHA decomposes and is irreversibly hydrolysed to form 2,3-diketoglyonic acid (Duarte & Lunec, 2005).

Figure 2: The reversible oxidation of ascorbate to dehydroascorbate through the ascorbate free radical, where one electron is delocalized across three of the oxygen atoms (Duarte & Lunec, 2005).

Ascorbate is brought into cells through sodium-coupled vitamin C transporter 1 (SVCT1) and SVCT2 (Chothe et al., 2013); however, vitamin C can be brought into cells faster in its oxidized DHA form via facilitated diffusion through the glucose transporters (GLUT). Cancer cells have an increased number of GLUT1 transporters than normal cells (Kang et al., 2002), and thus bring in an increased amount of DHA. Vitamin C in its DHA form acts as a pro-oxidant inside the cells mediating Fenton reactions with iron molecules and forming hydrogen peroxide and hydroxyl radicals (Di Tano et al., 2020; Duarte & Lunec, 2005; Schoenfeld et al., 2017).

Normal cells have sufficient antioxidant levels to reduce DHA back to ascorbate, have catalase (CAT) to safely convert hydrogen peroxide to water and oxygen, and have ferritin and
transferrin metal binding proteins to control the intracellular iron levels. However, most cancer cells have abnormally low levels of catalase and cannot neutralize the hydrogen peroxide and also have abnormally high amounts of redox active iron due to increased iron uptake and decreased iron export, thus making them selectively susceptible to the pro-oxidant effect of vitamin C and leading to increased oxidative stress and cell death (Doskey et al., 2016; Oberley, 1997).

In addition to the production of oxidative stress, other anti-cancer mechanisms of ascorbate include DNA damage, disrupting glycolysis at triose phosphate isomerase, decreasing adenosine triphosphate (ATP) production, mTOR inhibition, and triggering cell death through autophagy (Du et al., 2010; Fukumura et al., 2012; Ghanem et al., 2021; Y. Ma et al., 2014). The Warburg effect describes the phenomenon observed in cancer cells where energy metabolism occurs mainly through glycolysis rather than oxidative phosphorylation (Luengo et al., 2021; Seyfried et al., 2014; Zuberek et al., 2015).

Many cancer patients, especially those with late stage cancers, are deficient in vitamin C; with the patients with lower blood plasma levels having a shorter survival time (Ichim et al., 2011; Mayland et al., 2005; van Gorkom et al., 2019). Normal blood plasma vitamin C levels are tightly controlled around 70 – 85 μM in healthy people with a good diet, and this can be raised through oral supplementation only to around 100 – 220 μM (for taking 3 g every 4 hours) (Padayatty et al., 2004). However, a higher blood concentration level cannot be achieved through oral treatment due to limitations on the amount that can be absorbed in the intestinal tract. Unfortunately, this is not a sufficient level for treatment of cancer where blood plasma concentrations in the 0.3 – 15 mM range are necessary (Chen et al., 2005), and pharmacologically relevant treatments sufficient to kill cancer cells used in a clinical trial
resulted in blood plasma concentrations of 10 - 32 mM (Monti et al., 2012). This level of vitamin C in the blood only takes a few hours to decrease, with a half-life of around 2 hours, first-order elimination kinetics, excretion through the urine, and a return to normal blood plasma concentrations approximately 16 hours after the completion of the intravenous treatment (Lykkesfeldt & Tveden-Nyborg, 2019; Stephenson et al., 2013).

In vitro and in vivo work has shown that concentrations around 1 mM are needed to kill cancer cells (Chen et al., 2005; Padayatty et al., 2004); and mM treatment levels of vitamin C selectively kill cancer cells while being non-toxic to normal cells (Chen et al., 2005; Du et al., 2010). Thus, intravenous treatment is necessary since it can raise the blood plasma levels many times higher what is achievable through oral treatment (Duarte & Lunec, 2005; Padayatty et al., 2004).

It was not initially known that intravenous vitamin C is needed to be effective against cancer and raise the blood serum level to the concentration needed. Initial studies by Cameron and Campbell in 1974 reported promising results in 50 patients who had terminal cancer that had advanced to such an extent that it was untreatable by conventional methods. These patients were treated with 10 – 45 g vitamin C a day either intravenously, orally, or both, and 5 of these patients experienced tumor regression (Cameron & Campbell, 1974). Dr. Linus Pauling joined Dr. Cameron, and they expanded the next study to include 100 patients and again saw positive results, with the patients experiencing a much longer survival time of 293 days compared to the records of 1000 historical patients who had not received the vitamin C treatment and experienced an average survival time of 38 days (Cameron & Pauling, 1978). These promising results using a non-toxic therapy gained much attention at the time, and attempts were made to verify the claims. Two Japanese hospitals began treating their terminal cancer patients with a combination
of both oral and intravenous low-dose vitamin C (4 g per day or less) or high-dose vitamin C (5 g or more per day generally consisting of 6 – 12 g orally and 10 – 20 g intravenously, and an average of 25 g per day) and confirmed the increase in survival time and improvements in quality of life with high-dose vitamin C treatment found by Dr. Cameron, Dr. Campbell, and Dr. Pauling (Murata et al., 1982). In fact, the patients in the Fukuoka Torikai Hospital given high-dose vitamin C survived 5.6× longer than those given only low doses or no vitamin C (246+ days vs. 43 days), had a 3× lower death rate, and had improved appetite, mental alertness, and less pain. The Japanese doctors did not conduct double-blind trials as they were unable to exactly match pairs of patients and felt it would be unethical to exclude patients from treatment they found beneficial in their clinical experience (Murata et al., 1982). However, the Mayo clinic conducted two double-blind clinical trials with no success due to the unfortunate mistake of using only oral treatment rather than the combination of oral and intravenous treatment that was used in the initial studies. After the first clinical study showed no benefit in patients previously treated with chemotherapy (Creagan et al., 1979), the second clinical study was done with patients who had not previously been treated with chemotherapy since they had advanced colon cancer for which no chemotherapy was effective, and it was thought that the use of cytotoxic drugs might have decreased the effectiveness of the vitamin C treatment (Moertel et al., 1985). After the failure of this second clinical trial to show any benefit, the main-stream medical system has dismissed vitamin C treatment, despite controversy and mounting evidence for the benefit and safety of intravenous vitamin C in increasing the effectiveness of radiation and chemotherapy treatments while at the same time decreasing or at the very least not adding to the detrimental side effects (Böttger et al., 2021; Chiang et al., 1994; Di Tano et al., 2020; Du et al., 2015; Hoppe et al., 2021; Klimant et al., 2018; Kurbacher et al., 1996; Ma et al., 2014; Monti et
al., 2012; Nabzdyk & Bittner, 2018; Nauman et al., 2018; Ngo et al., 2019; Stephenson et al., 2013; van Gorkom et al., 2019; Yuen et al., 2018). Impressively, even low dose intravenous vitamin C of 2.5 g has been found to decrease pain and increase the average survival time in patients with radiotherapy-resistant bone metastasis from 2 months for the control group patients treated either with chemotherapy or nothing, to 10 months in the patients treated with vitamin C (Günes-Bayir & Kiziltan, 2015). Ascorbate treatment has even been found to partially restore drug sensitivity to imatinib-resistant cancer cells in vitro through inhibition of antioxidant production through nuclear factor erythroid 2-related factor 2 (Nrf2) activation of the antioxidant response element (ARE) (Tarumoto et al., 2004).

However, care must be taken when giving high-dose vitamin C to ensure the patient has good renal function for excretion and does not have hemochromatosis or deficiency in glucose-6-phosphate dehydrogenase, which leads to excessive and detrimental levels of oxidative stress and may result in homolysis (Khoshnam-Rad & Khalili, 2019).

1.9 Cellular Detoxification Pathways

There are two primary detoxification pathways for cellular detoxification and stress response in cells. The first pathway involves the nuclear factor-erythroid 2 like transcription factors, which induce various xenobiotic-metabolizing and antioxidant enzymes through activation of the antioxidant response element/electrophile response element (EpRE/ARE). The second main detoxification pathway involves the aryl hydrocarbon receptor (AhR), which induces various cytochrome P450 enzymes through the dioxin response element (DRE). The effects of nAg on aspects of both of these pathways were examined in this work.
The nuclear factor-erythroid 2 like transcription factors are from the cap’n’collar (CNC) family of transcription factors which respond to various cellular stresses and inflammation, and include nuclear factor-erythroid 2 like 1 (Nrf1), Nrf2, and Nrf3. These transcription factors bind to the ARE in genes at the consensus sequence of 5’-TGACNNNGC-3’, with N representing any nucleotide, through their CNC domain (Bugno et al., 2015). This sequence is found in the enhancer region of promoters for the genes of enzymes involved in stress response and detoxification such as SOD, glutathione S-transferase, glutathione-peroxidase, CAT, peroxiredoxins, heme oxygenase 1 (HO-1), and NAD(P)H-quinone oxidoreductase (Kubo et al., 2017; Zhang et al., 2009).

Nuclear factor-erythroid 2 like 1 is initially localized to the endoplasmic reticulum (ER) membrane after its translation at the rough ER. In its inactive form, the transactivation domain of Nrf1 is glycosylated, with this form of Nrf1 having a molecular weight of 120 kDa. Activation occurs when Nrf1 is retrotranslocated into the cytoplasm allowing deglycosylation and proteosomal proteolytic processing to occur resulting in the active Nrf1 form with a molecular weight of 95 kDa. Various truncated forms of Nrf1 are also produced, with these having distinct roles in the regulation of ARE gene expression. Active Nrf1 is translocated to the nucleus where it undergoes dimerization with small Maf protein and binds to the AREs, activating antioxidant and detoxification gene transcription (Bugno et al., 2015).

The transcription factor nuclear factor-erythroid 2 like 2 (Nrf2) is constitutively expressed at high levels but kept at low basal levels in the cytoplasm of the cell through ubiquitination by the Cullin3 (Cul3) ubiquitin E3 ligase complex and subsequent degradation by the proteosome. The kelch-like ECH-associated protein 1 (Keap1) acts as a bridge and keeps Nrf2 in close contact with Cul3. Most compounds that activate the ARE pathway are
electrophilic and bind to thiol groups, and Keap1 contains 27 cysteine residues enabling it to act as a sensor for these compounds (C. Hu et al., 2011). Upon exposure to ARE inducers, Nrf2 is released from Keap1 and Cul3 and translocates to the nucleus where it undergoes dimerization with small Maf protein and binds to the AREs, activating antioxidant and detoxification gene transcription (Hu et al., 2011). Antioxidant response element inducers include various toxins, the synthetic antioxidant TBHQ, or compounds found naturally in food such as SFN (from cruciferous vegetables), quercetin (from green tea, black tea, onions, and apples), and curcumin (from the spice turmeric) (Eggler et al., 2008).

Both Nrf1 and Nrf2 are known to be activated by tert-butylhydroquinone (TBHQ), a electrophilic quinone that is a synthetic antioxidant and is used as a food preservative (Chepelev et al., 2011; Eggler et al., 2008; Zhang et al., 2009). Sulforaphane (SFN) is a naturally produced phytochemical and potent antioxidant compound found in all cruciferous vegetables, especially in broccoli, which activates the cellular antioxidant response mainly through Nrf2 (Houghton et al., 2016; Hu et al., 2011; Kensler et al., 2013; Kubo et al., 2017).

The DRE pathway is induced by compounds such as halogenated aromatic hydrocarbons, which are very toxic and persistent environmental contaminants. The most potent and toxic halogenated aromatic hydrocarbons is 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) (Baston & Denison, 2011). These compounds strongly bind to the aryl hydrocarbon receptor (AhR) transcription factor, upon which AhR translocates to the nucleus where it undergoes dimerization with the nuclear protein Ah receptor nuclear translocator (ARNT) and can then bind to the DRE and induce gene expression, such as for the cytochrome P450 (CYP) 1 family of detoxification enzymes (He et al., 2011; Vorrink et al., 2014).
1.10 Cellular Response to Hypoxic vs Normoxic Conditions

Hypoxia can occur in the body if the levels of oxygen are restricted due to a medical reason such as cardiac arrest or stroke, during embryogenesis, or in solid tumors. Low oxygen may also be experienced by humans at high altitude (Ozer & Bruick, 2007).

Under normal oxygen (normoxic) conditions, hypoxia-inducible factor-1α (HIF-1α) is continuously hydroxylated on Pro564 by oxygen-dependent PHD enzymes, leading to the recognition of HIF-1α by E3 ubiquitin ligases, and subsequent proteasomal degradation (Osipyants et al., 2018). In low oxygen conditions, HIF-1α hydroxylation and degradation is inhibited, and HIF-1α travels to the nucleus where it then binds to HIF-1β (ARNT). This heterodimer then binds to the hypoxic response elements (HRE) in the promoters of HIF target genes (such as erythropoietin, vascular endothelial growth factor, inducible nitric oxide synthase, etc.), activating gene expression involved with cellular hypoxic response and cellular adaptation to the stress (Ozer & Bruick, 2007; Vorrink et al., 2014; Yang et al., 2016). L-ascorbate functions as a reducing agent ensuring PHD activity as well as serving as a co-substrate in the active site (Osipyants et al., 2018). Prolyl hydroxylases require ascorbic acid as a reducing agent and co-substrate in the active site in order to perform their many functions, such as to hydroxylate HIF-1α or collagen.

1.11 Cellular Response to ER Stress

The ER is an organelle with many functions including protein synthesis and folding, lipid biogenesis, and calcium storage. It forms a reticular network throughout the cytosol, and interacts closely with the mitochondria for the transfer of various proteins, ions, and lipids (Kim et al., 2008; Li et al., 2019). Misfolded and damaged proteins may build up in the ER under
conditions of cellular oxidative, hypoxic, viral, toxin, environmental, or starvation stress; resulting in ER stress (Christen & Fent, 2012; Kim et al., 2008; Simard et al., 2015). Unfolded protein response (UPR) signalling attempts to respond to this situation by increasing protein degradation and the production of chaperones, however, if the damage is too great ER stress may lead to cell death (Christen et al., 2013; Fulda et al., 2010). Three ER membrane spanning proteins, protein kinase RNA-like ER kinase (PERK), activating transcription factor-6 (ATF-6), and inositol-requiring enzyme-1α (IRE-1α); are activated in response to ER stress. These proteins are inactive when bound to the ER chaperone GRP78, and become active when GRP78 dissociates during ER stress to assist in protein folding (Christen & Fent, 2012; Simard et al., 2016).

The UPR pathway through PERK involves activation of eukaryotic translation initiation factor 2 (eIF2-α) leading to inhibition of protein translation. Under conditions of persistent ER stress, eIF2-α increases the translation of activating transcription factor 4 (ATF-4), which regulates many genes integral to cell survival, cellular stress response, or apoptosis (Christen & Fent, 2012; Murley & Nunnari, 2016; Rozpędek et al., 2016; Simard et al., 2016; R. Zhang et al., 2012).

In the UPR pathway through ATF-6, ATF-6 is transported to the Golgi apparatus and activated by proteolytic cleavage, releasing the N-terminal fragment which acts as a transcription factor to stimulate gene expression either for cell survival such as through X-box binding protein 1 (XBP1) or ARF-4, or for cell death (Fulda et al., 2010; Simard et al., 2015, 2016).

Active IRE-1α excises an intron from XBP1 mRNA allowing it to be translated into XBP1 protein, which is a transcription factor that induces the expression of various target genes such as Grp78. IRE-1 can also interact with many other proteins and mRNA, modulating protein
synthesis, inflammatory pathways, and apoptosis (Christen & Fent, 2012; Gerakis & Hetz, 2018; Simard et al., 2016; Zhang et al., 2012).

1.12 Cellular Oxidative Stress

Oxidative stress occurs in biological organisms when there is an imbalance with too many ROS for the available antioxidants to neutralize. These include any form of oxygen with an unpaired electron in its outer electron orbital such as superoxide, hydrogen peroxide, the hydroxyl radical, singlet oxygen, alkoxy radicals, peroxy radicals, hydrochlorous acid, and hypobromous acid (Chen et al., 2010). In the mitochondria, elections may escape from the electron transport chain and bind to $O_2$ forming superoxide, which may be further converted to hydrogen peroxide or hydroxyl radicals. Inhibition of oxidative phosphorylation results in decreased ATP production and increased oxidative stress through the production of superoxide (Asharani et al., 2009).

Oxidative stress can be neutralized in biological systems through various enzymes (superoxide dismutase, catalase, and peroxidase), various peptides or proteins (glutathione, ferritin, and transferrin), cofactors (NADPH and thioredoxin), and vitamins (vitamins E, C, and A) (Duarte & Lunec, 2005). Superoxide dismutase neutralizes superoxide and converts it to oxygen and hydrogen peroxide, which is further neutralized to water and oxygen by CAT. Glutathione is a tripeptide made up of glutamic acid, cysteine, and glycine; and is the main cellular antioxidant and detoxification molecule (Gaucher et al., 2018). The availability of cysteine limits the rate of glutathione synthesis, thus supplementation with NAC supplies the rate-limiting amino acid and increases intracellular glutathione production (McCarty et al., 2018). Additionally, increased supplementation with glycine is beneficial in increasing
glutathione synthesis (McCarty et al., 2018). Low levels of intracellular ROS function as important signalling molecules; however, high levels of ROS are damaging and may overwhelm the cellular antioxidant defenses resulting in glutathione depletion, activation of the cellular antioxidant response, oxidative damage to mitochondria, proteins, or DNA, and ultimately cell death (Asharani et al., 2009; Blanco et al., 2018; Gao et al., 2017; McShan et al., 2014; Zhao et al., 2016a).

It is commonly reported in the literature that the mode of action of nAg toxicity is mainly due to the production of silver ions and resulting ROS in the cell (Asharani et al., 2009; Blanco et al., 2018; Chorley et al., 2014; Farah et al., 2016; Gao et al., 2017; Jeong et al., 2016; Kang et al., 2012; Y. H. Lee et al., 2014; Manshian et al., 2015; Oh et al., 2016; Prasad et al., 2013b; Zhang et al., 2015; Zhang & Gurunathan, 2016). However, the amount of ROS produced by nAg is in a large part determined by the dose, treatment time, and size of nAg used, as well as by the different sensitivities of the various cell lines (Gao et al., 2017; McShan et al., 2014; Oh et al., 2016; Zhao et al., 2016b; Zhu et al., 2017). Additionally, no change or a decrease in ROS has been found with nAg treatment in HepG2 and human colon cancer Caco-2 cells (Sahu et al., 2014), human neutrophils from healthy donors (Poirier et al., 2016), HEK293T cells (Chen et al., 2014b), human gingival fibroblasts (HGFs) (Gallorini et al., 2016), rat liver mitochondria (Pereira et al., 2018), ToxTracker mouse embryonic stem cell lines (Karlsson et al., 2014), and mouse erythroleukemia (MEL) cells (Qian et al., 2015; Wang et al., 2013).

Intracellular ROS production is commonly measured using the non-polar dye 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Manshian et al., 2015). However, this may not provide reliable results and the species of ROS measured using this dye are unknown (Xiuping Chen et al., 2010; Lee et al., 2014; Zhang et al., 2015). Detecting ROS through means
of a fluorescent dye and flow cytometry is more reliable and gating can be performed to remove background fluorescence and cell debris (McBee et al., 2017). CellROX fluorescent probes are used for detecting superoxide and hydroxyl radicals, with CellROX Green Reagent able to detect ROS in the nucleus and mitochondria and CellROX Deep Red and CellROX Orange reagents able to detect ROS the cytoplasm. Resorufin is a fluorescent dye specific for H$_2$O$_2$ (Choi et al., 2015). The dihydroethidium (DHE) dye is able to detect superoxide (Asharani et al., 2009).

1.13 Cellular WNT Signalling Pathways

Wingless/Integrated-1 (WNT) proteins are lipid-modified glycoproteins, and function in WNT signaling to regulate stemness as well as many developmental processes such as cell proliferation, cell differentiation, organogenesis, and the regeneration of tissue (Jung & Park, 2020; Nygaard et al., 2021; Zhan et al., 2017). The WNT name comes from a combination of the names for the wingless gene in Drosophila melanogaster and its murine equivalent Integrated-1 (Int-1), a proto-oncogene which is now called Wnt-1, and the human homolog of which is WNT-1 (Nusse et al., 1991).

After translation, the WNT proteins are modified by palmitoylation in the ER by porcupine (PORCN), making them hydrophobic and unable to cross cellular membranes except with the help of carrier proteins. Porcupine is an ER membrane-bound O-acyltransferase which catalyzes the addition of a mono-unsaturated palmitoleate, specifically palmitoleyl-coenzyme A (CoA), to a serine residue on the WNT proteins. Once palmitoleated, the WNT proteins bind to the membrane transport protein WNTless, which carries WNT from the ER to the plasma membrane for secretion (Nygaard et al., 2021). Further post-translational modification of WNT occurs by glycosylation in the Golgi apparatus on its way to the plasma membrane. After
delivering the WNT protein to the plasma membrane, WNTless returns to the ER to repeat the process. The WNT proteins function as short-range signalling molecules, and once at the plasma membrane they are transported through facilitated diffusion to the appropriate Frizzled receptor on a nearby cell, stimulating WNT signalling (Nygaard et al., 2021). WNT signalling has been separated into β-catenin-dependent and -independent signalling pathways, although it is now thought that these pathways intertwine and may both be activated during tumor formation.

The β-catenin-dependent pathway is termed canonical, and involves WNT proteins, such as WNT3A and WNT1, and binds to Frizzled receptors which leads to the inactivation of the β-catenin destruction complex, thus allowing β-catenin to accumulate and translocate to the nucleus where it functions as a transcriptional co-regulator in the induction of gene expression involved in cell proliferation and differentiation (Cooper et al., 2019).

In the β-catenin-independent pathways (also termed non-canonical or WNT/planar cell polarity[PCP] and calcium pathways), WNT proteins such as WNT5A bind to Frizzled receptors and activate gene expression independently of β-catenin (Zhan et al., 2017). Over-activation of WNT signaling has been found to result in tumor formation, particularly colon cancer; however, the role of WNT signaling in cancer is very complex and not well understood. Hereditary colon cancer has been found to result from mutations in the adenomatous polyposis coli (APC) gene, and approximately 70% of all colon cancers have APC mutations. These mutations result in loss of APC function and abnormally high T-cell factor (TCF)4/β-catenin signaling (Jung & Park, 2020).
1.14 Mitochondrial Function and Apoptosis

Mitochondria are complex organelles that form a complex reticular network throughout the cytosol, facilitating energy production, cellular function, and communication between the cellular organelles (Giorgi et al., 2009; Sukhorukov et al., 2012). They perform many functions including ATP production, the generation of body heat, and provide a location for intracellular calcium stores and are involved in calcium signalling (McGuire, 2019). Mitochondria have the vital function of sensing the level of cellular stresses such as from nutrient deficiency or viral infection, and respond by regulating cellular danger signalling pathways and remodelling the mitochondrial network to adapt and combat the stress, undergoing mitochondrial fission and fusion in response to the cellular needs (Galluzzi et al., 2012). Fusion and increased number of mitochondria facilitates increased energy production which is needed for faster growing cells or cells with higher energy requirements. Organs including the heart, brain, liver, and muscle all require large amounts of energy to function optimally, with egg cells having the highest energy need during mitosis (Li et al., 2020). However, high levels of intracellular stress and damage may cause excessive mitochondrial fission and damage, which if not repaired through fusion to healthy mitochondria or removal through autophagy, results in the release of pro-apoptotic proteins leading to cell death (Ma et al., 2020).

Mitochondrial mediated intrinsic apoptosis is a complex pathway that involves p53 inhibition of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) proteins resulting in mitochondrial outer membrane permeabilization and releasing cytochrome C as well as other mitochondrial proteins into the cytosol (Endo et al., 2006; Wang & Youle, 2009). The disintegration of the mitochondria is the point of no return in intrinsic apoptosis; and released cytochrome C binds to apoptotic protease-activating factor-1 (Apaf-1) in the cytosol, which undergoes ATP or
deoxyadenosine triphosphate (dATP) dependent oligomerization forming the apoptosome complex. In the intrinsic apoptosis pathway, the apoptosome activates initiator caspase-9, which activates executioner caspase-3 and -7, resulting in caspase signalling cascades, and ultimately resulting in DNA fragmentation, cell death, and externalization of phosphatidylserine, which is the signal for degradation and removal via phagocytosis (Saito et al., 2006). Second Mitochondria-Derived Activator of Caspase (Smac) and Omi are proteins that are also released from the mitochondria, and once in the cytosol they bind to Inhibitor of Apoptosis Proteins (IAPs), preventing them from inhibiting activation of the caspases (Galluzzi et al., 2012; Giampazolias et al., 2018; Wang & Youle, 2009).

Alternatively, extrinsic apoptosis does not involve the mitochondria and is a pathway where death receptors bind to capsase-8 which in turn activate the executioner caspase-3 and caspase-7 (Murley & Nunnari, 2016; Sukhorukov et al., 2012; Zhang et al., 2015). Nanosilver treatment has been found to activate both intrinsic and extrinsic apoptosis in HCT116 cells (Dasgupta et al., 2018), HepG2 cells (Xue et al., 2018), MCF-7 cells (George et al., 2018), and rat pheochromocytoma (PC12) cells (Hadrup et al., 2012). Additionally, nAg treatment of rats via intratracheal instillation has been found to adversely affect the mitochondria in the lung tissue, with swollen mitochondria, nAg visible inside the mitochondria, increased fission and decreased fusion, and apoptosis through caspase-3 (Ma et al., 2020).

1.14.1 Mitochondrial Oxidative Phosphorylation and Glycolysis

Oxidative phosphorylation in the mitochondria is used for energy production in healthy cells. Electrons obtained from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) are passed along the electron transport chain complexes, at the same time
as complex I (NADH-coenzyme Q oxidoreductase), complex III (coenzyme Q-cytochrome c oxidoreductase), and complex IV (cytochrome c oxidase) form a proton gradient across the inner mitochondrial membrane. This accumulation of protons in the intermembrane space forms the proton motive force (PMF) needed to power ATP production by complex V (F1F0-ATPase), as complex V transfers the protons along the concentration gradient back into the mitochondrial matrix (Vafai & Mootha, 2012). Complex I transfers electrons from NADH while complex II (succinate-coenzyme Q reductase or succinate dehydrogenase) transfers electrons from FADH2. Complex I and II both transfer electrons to Coenzyme Q which changes between its oxidized ubiquinone form and its reduced ubiquinol form. Coenzyme Q in its ubiquinol form passes electrons to complex III, which in turn passes the electrons to complex IV via cytochrome C, and molecular oxygen is the final electron acceptor at complex IV and forms water. If energy production by the electron transport chain in the mitochondria is inhibited or damaged, cells may adapt and produce energy through glycolysis, although this is a much less efficient process and only produces 2 ATP per molecule of glucose as compared to the 36 ATP produced per molecule of glucose by oxidative phosphorylation (Kim et al., 2009; Yetkin-Arik et al., 2019).

This shift to using glycolysis for energy production has been indicated with non-toxic nAg treatment in HepG2, HeLa, renal carcinoma A498, PC3, and HEK293T cells, where the levels of the glycolytic intermediates lactate and pyruvate increased, along with indications of mitochondrial dysfunction including impaired ATP production levels, decreased mitochondrial membrane potential, and inhibited lipid metabolism (Chen et al., 2014b). Decreased mitochondrial membrane potential or decreased ATP production as a result of nAg treatment has also been found in HCT116 (Satapathy et al., 2015), A549 (Jeong et al., 2016), SH-SY5Y (Li et al., 2018b), A2780 (Zhang & Gurunathan, 2016), rat cerebellar granule cells (CGC) (Ziemińska
et al., 2014; Ziemińska & Strużyńska, 2016), HepG2, and Caco-2 cells (Sahu et al., 2014). *In vivo*, impaired energy production as well as mitochondrial swelling, DNA fragmentation, and increased apoptosis has been observed in the liver tissue of rats treated with nAg (Lee et al., 2013; Pereira et al., 2018).

### 1.14.2 Measuring Mitochondrial Oxidative Stress

In this work, mitochondrial oxidative stress in response to nAg and AgNO$_3$ treatment was measured using MitoSOX. MitoSOX is a derivative of dihydroethidium with a positively charged triphenylphosphonium group added. MitoSOX is targeted to the mitochondria and accumulates there due to its positive charge, thus allowing MitoSOX to detect mitochondrial oxidative stress. The dihydroethidium part of MitoSOX is oxidized by mitochondrial ROS to 2-hydroxyethidium, which binds to mitochondrial DNA and becomes fluorescent with an excitation at 400 nm and an emission at 590 nm. Since the main ROS in the mitochondria is superoxide, MitoSOX mainly detects superoxide levels in the mitochondria (Kauffman et al., 2016; Monteiro et al., 2020; Mukhopadhyay et al., 2007).

Care must be taken to analyze the stained cells quickly, since stained cells that are left more than about 30 – 40 min after the initial staining period may experience disrupted mitochondrial structure or disrupted mitochondrial membrane potential gradient in apoptotic cells. This releases the MitoSOX stain and allows it to bind to the nuclear DNA and exhibits strong nuclear staining (Mukhopadhyay et al., 2007; Sundqvist et al., 2017).
1.15 Objective and Rational

Nanosilver has recently shown promising results when combined with radiation treatment. Combined treatment of rats with glioma with intratumoral nAg followed by a single dose of radiation increased the life span by 513.2%, which was much higher than that seen with either treatment on its own (Liu et al., 2013). Additionally, it has been found that the production of ROS is involved in the radiosensitization of cancer cells by nAg (Wu et al., 2016). Radiation is a pro-oxidant cancer therapy, as is vitamin C. Thus, due to the benefit of combining a pro-oxidant therapy with nAg treatment, and the benefit that vitamin C has shown in combination with other cancer treatments, this research investigated combined nAg and sodium ascorbate treatments.

Since oral ingestion brings nAg and ascorbate into the gastrointestinal tract, it is feasible that oral administration could be used to bring nAg to or near the site of concern for colon cancer. Both nAg and ascorbate have shown promising anti-cancer effects, although much remains to be discovered. In this work, HCT116 colon cancer cells were used to examine the potential beneficial or detrimental effects of nAg and sodium ascorbate on colon cancer. HCT116 cells are large intestine epithelial cells that are commonly used in such studies. More than 85% of colon cancers have modulated WNT/β-catenin signalling (Proto et al., 2017), which is also found in HCT116 cells (Sekine et al., 2002). In this work, HIEC-6 non-cancer colon cells were used as a control to examine the effects of nAg and sodium ascorbate on non-cancer colon cells. HIEC-6 cells are small intestine epithelial cells that are commonly used as a non-cancer colon cell line in such studies.

The aim of this work is to examine the biological effects of nAg, sodium ascorbate, and combined nAg and sodium ascorbate treatments on HCT116 and HIEC-6 cells; examining the effects on cell cycle, antioxidant response pathways, dioxin response pathways, WNT signaling
pathways, cellular and mitochondrial oxidative stress, and mitochondrial function at sub-toxic treatment levels.

The experimental design and techniques used in this work are depicted in Figure 3. Cell viability with the various treatments was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry was used to measure the levels of cellular and mitochondrial oxidative stress, various aspects of ER stress, and to examine effects on the cell cycle. Western blotting was used to examine protein expression, while qRT-PCR and luciferase reporter gene assays were used to examine gene expression. The Seahorse assay was used to examine mitochondrial function (see Figure 3).

**Figure 3**: Schematic diagram of the experimental design. Created using BioRender.com.
Chapter 2: Materials and Methods

2.1 Chemicals and Reagents

Polyvinylpyrrolidone (PVP)-coated and citrate-coated 10 nm nanosilver spheres (1 mg/mL) were obtained from NanoComposix (Fortis Life Sciences, San Diego, CA, USA). The nanosilver was synthesized using a seeded aqueous growth method starting with aqueous metal salts and purified using centrifugation and filtration methods. CellROX and MitoSOX were obtained from Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Pierce RIPA Buffer was obtained from Thermo Scientific (Rockford, IL, USA). Sodium Pyruvate, Glucose Solution, and L-Glutamine were obtained from Gibco (Thermo Fisher Scientific, Waltham, MT, USA).

Rotenone, antimycin A, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), trichloroethanol (TCE), 5-bromo-2′-deoxyuridine (BrdU), sodium ascorbate (SA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), N-acetyl-L-cysteine (NAC), polyethylenimine (PEI), tert-butylhydroquinone (TBHQ), propidium iodide (PI), thapsigargin, urea, sodium azide, Triton X-100, and potassium chloride (KCl) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Dithiothreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1× Protease Inhibitor, luciferin, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), adenosine triphosphate (ATP), bovine serum albumin (BSA), glycine, Tris, glycylglycine, magnesium sulfate (MgSO₄), potassium dihydrogen phosphate (KH₂PO₄), sodium dodecyl sulfate (SDS), and Tween 20 were obtained from BioShop Canada Inc. (Burlington, ON, Canada).
Silver nitrate (AgNO₃) was obtained from Engelhard Industries (NJ, USA). Sulforaphane (SFN) was obtained from the Cayman Chemical Company (Ann Arbor, Michigan, USA). 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) in toluene (catalog number: D-404S) was obtained from AccuStandard (New Haven, CT, USA). Nonidet P40 Substitute (NP-40) was obtained from USB Corporation (Cleveland, OH, USA). RNaseA was obtained from Boehringer Mannheim Pharmaceuticals Corporation (Gaithersburg, MD, USA). Dimethylsulfoxide (DMSO) was obtained from BDH (VWR International LLC Radnor, PA, USA). Cumene hydroperoxide Solution (H₂O₂) was obtained from Fluka (Steinheim, Germany). Disodium hydrogen phosphate (Na₂HPO₄) was obtained from JT Baker (NJ, USA). Magnesium chloride (MgCl₂) was obtained from Mallinckrodt, Inc. (Paris, Kentucky, USA). Potassium dihydrogen phosphate (KH₂PO₄) was obtained from Analar (NJ, USA). Methanol was obtained from Anachemia (VWR International, Mississauga, ON, Canada). Chlorophenol red-β-D-galactopyranoside (CPRG) was obtained from Roche (Rotkreuz, Switzerland).

2.2 Nanoparticle Characterization

2.2.1 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) analysis of the nAg was done by placing a drop of the 10 nm PVP nAg on a TEM 300 mesh copper grid covered with FORMVAR carbon support film (Electron Microscopy Sciences, PA, USA). The nAg on the grid was allowed to dry and the TEM analysis performed (thanks to Dr. Jianqun Wang, Nano Imaging Facility, Carleton University, Ontario, Canada).
2.2.2 Ultraviolet/Visible (UV-Vis) Spectrum

The quality of the nAg was periodically checked by assessing its ultraviolet/visible (UV-Vis) spectrum and ensuring that the absorbance peak size and shape did not change due to aggregation. One millilitre ddH₂O was placed in a quartz cuvette as the blank and the baseline measurement using a Cary 100 Bio UV-Visible Spectrophotometer. Five microliters of nAg was then added to the ddH₂O blank and the absorbance measured across 200 – 800 nm wavelengths.

2.2.3 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The 10 nm PVP-nAg was diluted in 7 mL Dulbecco’s Modified Eagle Medium (DMEM) media with 5% FBS and 5% NCS to 1, 5, and 10 µg/mL and incubated at 37°C and 5% CO₂ for 0, 6, and 24 hours in a cell culture dish. The solution was then transferred to a centrifuge tube and centrifuged at 25400 rpm for 2 hours at 4°C in a Beckman Type 100 Ti centrifuge. The time taken to transfer the samples into the centrifuge tubes and seal the tubes before centrifugation was approximately 1 hour at room temperature, and thus the time before the nanoparticles were removed from the media by centrifugation was 1, 7, and 25 hours. The media supernatant was removed being careful to not disturb the nanoparticle pellet at the bottom. A silver ion standard curve was made with AgNO₃ in media at 0, 0.05, 0.1, 1, 2, and 5 µg/mL AgNO₃. The media supernatant samples were acidified with 1% nitric acid and the silver ion content of the media measured with ICP-MS (thanks to Dr. Alexandre J. Poulain, University of Ottawa, Core Molecular Laboratory, Ontario, Canada). The silver ion standard curve \( y = 644.41x - 57.72 \), where \( x \) is the AgNO₃ concentration, and \( y \) is the silver ion concentration) was used to determine the AgNO₃ equivalent concentration from the silver ion content measured in the media supernatant samples for 1, 5, and 10 µg/mL PVP-nAg incubated in media for 1, 7, and 25 hours.
The incubation time versus the AgNO₃ equivalent concentration was graphed for each PVP-nAg concentration, a polynomial curve of best fit used, and the equation of the curve used to estimate the AgNO₃ equivalent concentrations with an incubation time of 4-hours. The percent increase in AgNO₃ equivalent concentration from 5 to 10 µg/mL PVP-nAg was used to estimate the 4-hour AgNO₃ equivalent concentration of 20 µg/mL PVP-nAg from 10 µg/mL PVP-nAg. Graphing the 4-hour AgNO₃ equivalent concentrations for 1, 5, and 10 µg/mL PVP-nAg, the equation of the curve of best fit (y = 0.5808x^0.3487, where x is the nAg concentration, and y is the equivalent AgNO₃ concentration at 4-hours) was used to estimate the AgNO₃ equivalent concentration for 30 µg/mL PVP-nAg incubated in media for 4-hours.

2.3 Cell Culture and Hypoxic Treatment

Cells were grown in normoxic conditions in a humidified incubator at 37°C with 5% CO₂ and 21% O₂ (Forma Series II Water Jacket CO₂ Incubator, Waltham, MA, USA). Hypoxia treated cells were treated in hypoxic conditions in a humidified incubator at 37°C with 5% CO₂ and 1% O₂ for 4 or 24 hours before harvest and harvested in hypoxic conditions using a hypoxic workstation (Baker Ruskinn InvivO 2 400 Workstation).

Human colon carcinoma HCT116 cells were grown in DMEM (Wisent Inc., QC, Canada) with 5% fetal bovine serum (FBS) and 5% newborn calf serum (NCS) (HyClone, UT, USA). The HCT116 cells used in the Seahorse assays were grown in McCoy’s 5A media (Wisent Inc., QC, Canada) with 10% FBS. The stably transfected HCT116-ATF4 and HCT116-XBP1 cell lines (Nougarède et al., 2018) (a kind gift from Dr. Bruce McKay, Carleton University, Ottawa, ON, Canada) were grown in DMEM with 5% FBS and 5% NCS.

Human intestinal epithelial cells 6 (HIEC-6) (a kind gift from Dr Yannick Benoit, Faculty of Medicine at University of Ottawa, Ottawa, ON, Canada) and were grown in OptiMEM
(Gibco, Thermo Fisher Scientific, Waltham, MT, USA) with 5% Premium FBS (Wisent Inc., QC, Canada), 10 mM HEPES (from 1M 100× stock, Gibco, Thermo Fisher Scientific, Waltham, MT, USA), 10 mM Glutamax (from 100× stock, Gibco, Thermo Fisher Scientific, Waltham, MT, USA), and 10 ng/mL Epidermal Growth Factor (Catalog #: PHG0311L, Gibco, Thermo Fisher Scientific, Waltham, MT, USA) (Benoit, 2018). HIEC-6 cells were harvested using TrypLE express (Gibco, Thermo Fisher Scientific, Waltham, MT, USA), and were used up to passage 30.

Human embryonic kidney (HEK 293T) cells and rat pheochromocytoma (PC-12) cells were grown in DMEM with 7.5% FBS and 2.5% NCS and were harvested using 1× trypsin. Mouse macrophage RAW264.7 cells were grown in DMEM with 7.5% FBS and 2.5% NCS RAW264.7 and were harvested by scraping. Human neuroblastoma SH-SY5Y cells were grown in a 1:1 mixture of DMEM and Ham’s F12 Nutrient Mixture (BioShop Canada, Burlington, ON, Canada) with 7.5% FBS and 2.5% NCS and were harvested using 1× trypsin (Gibco, Thermo Fisher Scientific, Waltham, MT, USA).

Cell pictures were taken on the EVOS M5000 (Thermo Fisher Scientific, Waltham, MT, USA).

2.4 MTT Cell Viability Assay

Cells were plated in a 96 well plate at 1.5 × 10^5 cells/mL for HCT116 cells, 2.0 × 10^5 cells/mL for HIEC-6 cells, 1.5 × 10^5 cells/mL for HEK 293T cells, 2.0 × 10^5 cells/mL for RAW 264.7 cells, 5.0 × 10^5 cells/mL for SH-SY5Y cells, and 2.0 × 10^5 cells/mL for PC-12 cells. The cells were incubated at 37°C and 5% CO₂ for 24 or 42 hours. The old media was removed, new media was added with the treatments, and the cells allowed to further incubate for a treatment
The treatment was removed, 100 µL new media added per well, and 10 µL MTT (5mg/mL in 1× PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄]) added to each well. Wells with cells treated with sodium ascorbate were rinsed once with 1× PBS before adding the new media and the MTT. The plate was incubated for 1-2 hours at 37°C and 5% CO₂. The media was completely removed and 50 µL DMSO added to solubilize the formazan crystals and the absorbance was measured at a wavelength of 570 nm with a background subtraction at 630 nm to correct for any differences in the plastic of each well (PowerWave XS microplate reader, BioTek Instruments, Winooski, Vermont). At least three wells of untreated cells were kept without MTT treatment, and the average absorbance of the DMSO from these wells was subtracted from the absorbance of each of the other wells to correct for background due to the presence of the cells. A minimum of three replicates were done, and the data analyzed with One-Way ANOVA and Two-Way ANOVA and the significance determined with Dunnett’s test.

2.5 CellROX

Cells were plated in a 24 well plate at 5.0 – 6.0 × 10⁴ cells/well for HCT116 cells, and 8.7 – 9.5 × 10⁴ cells/well for HIEC-6 cells. The cells were incubated at 37 and 5% CO₂ for 24 or 44 hours, and then treated with nAg and AgNO₃ and incubated for 24 or 4 hours respectively. The positive control was done with 25 µM H₂O₂ for 1 hour before the CellROX stain was added for a total incubation time of 2 hours. The negative control NAC pre-treatment was done for 2 hours with 5 mM NAC, then 25 µM H₂O₂ was added into the NAC treatment for 1 hour before the CellROX stain was added and incubated for a further hour. CellROX (5 µM) was added into the treated media and incubated for 1 hour before cell harvest and analysis by flow cytometry. The
wells were gently washed three times with sterile PBS, harvested with 100 μL 1x trypsin, and the trypsin stopped with media. The cells from the individual wells were transferred into Eppendorf tubes and the media was removed after centrifugation at 1,000 xg for 1 min, the cell pellet rinsed with PBS, and then resuspended in flow buffer (0.5% BSA and 2 mM EDTA in PBS). Untreated and unstained cells were heat shocked in a 45°C water bath for 5 min and then stained with 2 μL/mL PI to measure a live-dead peak. Cell samples were kept on ice and analyzed by flow cytometry (BD Accuri C6 flow cytometer).

Cell debris and dead cells were removed from analysis by plotting forward scatter area (FSC-A) vs side scatter area (SSC-A) and gating around the live cell population, and cell doublets were removed from the analysis of the live cells by plotting FSC-A vs forward scatter height (FSC-H) and selecting the singlets. A histogram of the FL4 channel vs count was done to find the mean FL4 fluorescence. The mean FL4 fluorescence of the untreated unstained sample was subtracted from all the samples, and the data was normalized to the untreated sample stained with CellROX. A minimum of three replicates was done, and the data analyzed with One-Way ANOVA and the significance determined with Dunnett’s test.

2.6 MitoSOX

Cells were plated in a 24 well plate at 5.0 – 6.0 × 10⁴ cells/well for HCT116 cells, and 8.7 – 9.5 × 10⁴ cells/well for HIEC-6 cells. The cells were incubated at 37°C and 5% CO₂ for 24 or 44 hours, and then treated with nAg and AgNO₃ and incubated for 24 or 4 hours respectively. The positive control was done with 5 or 15 μg/mL Antimycin A for 30 min before harvest, and with 25 μM H₂O₂ for 2 hours before harvest. The negative control NAC pre-treatment was done for 2 hours with 5 mM NAC before adding the positive control. MitoSOX stock (5 mM in DMSO) was diluted to 1 μM in PBS (MitoSOX was diluted in media for the HIEC-6 cells to
improve viability). The cells were gently washed with warm 37°C sterile PBS and harvested with trypsin. Media was added to stop the trypsin and the cells were transferred into Eppendorf tubes and the media removed after centrifugation at 1,000 xg for 1 min. The cell pellets were washed with PBS, then resuspended in 0.5 mL 1 μM MitoSOX and incubated in the incubator for 30 min covered with sterile tin foil. After the incubation, 0.5 mL PBS was added to the tubes, centrifuged at 1,000 xg for 2 min, and the solution removed. The cell pellets were washed with an additional 0.5 mL PBS. To reduce cell loss during the washes half the wash was removed and the sample re-centrifuged before removing the remainder of the wash. The samples were resuspended in 100 μL Flow Buffer (0.5% BSA and 2 mM EDTA in PBS) and kept on ice in the dark and run immediately on the flow cytometer (BD Accuri C6 flow cytometer).

Cell debris and dead cells were removed from analysis by plotting FSC-A vs SSC-A and gating around the live cell population, and cell doublets were removed from the analysis of the live cells by plotting FSC-A vs FSC-H and selecting the singlets. A histogram of the FL2 channel vs count was done to find the mean FL2 fluorescence. The mean FL2 fluorescence of the untreated unstained sample was subtracted from all the samples, and the data was normalized to the untreated sample stained with MitoSOX. A minimum of three replicates was done, and the data analyzed with One-Way ANOVA and the significance determined with Dunnett’s test.

2.7 Seahorse Cell Energy Phenotype Test and Cell Mito Stress Test Assay

Cells were plated in the 6 centre wells of an 8 well Agilent Seahorse XFp Cell Culture Miniplate with a total volume of 80 μL at 15,000 cells/well in McCoy’s media with 10% FBS for HCT116 cells and 20,000 cells/well in HIEC-6 cell culture media for HIEC-6 cells, to give an oxygen consumption rate (OCAR) reading between 20 – 160 pmol/min and an extracellular
acidification rate (ECAR) reading between 10 – 90 mPH/min. The remaining 2 wells were filled with 80 µL of cell culture media. The surrounding large wells on the Cell Culture Miniplate were filled with 400 µL PBS. The cells were allowed to sit in the hood for 15 min following plating and then incubated at 37°C and 5% CO₂ for 24 hours to allow adherence. The Agilent Seahorse XFp Sensor Cartridge was rehydrated with 200 µL rehydration buffer in each of the 8 centre wells with 400 µL rehydration buffer in the surrounding large wells and incubated at 37°C and 0% CO₂ for 24 hours.

For the Cell Energy Phenotype Test experiments, after 24 hours the cells were gradually transferred to Seahorse Assay Medium (XF Base Medium Minimal DMEM without Phenol Red [Agilent Technologies, Inc., Santa Clara, CA, USA] supplemented with 1 mM sodium pyruvate, 2 mM glutamine, and 10 mM glucose, pH 7.4) by removing 60 µL of the cell culture media from the wells of the Cell Culture Miniplate and replacing it with 60 µL Seahorse Assay Medium twice. All the media was then removed from the wells and replaced with 180 µL Seahorse Assay Medium. The Cell Culture Miniplate was incubated at 37°C and 0% CO₂ for 1 hour. The nAg and AgNO₃ treatments were loaded into Port A of the Sensor Cartridge at 10× the final concentration desired in the well and the cartridge was placed in the Seahorse analyzer 15 minutes before the end of the Cell Culture Miniplate incubation and the Seahorse analyzer allowed to calibrate. The Miniplate was then also placed in the Seahorse analyzer (Agilent Seahorse XFp) and the OCAR and ECAR stimulated by the injected treatments were measured over 6 hours.

For the Cell Mito Stress Test Assay, the cells were treated with 20 µg/mL nAg and 1.65 µg/mL AgNO₃ for 4 hours before the assay. The treatments were then removed and replaced with 180 µL Seahorse Assay Medium as above, and the Cell Culture Miniplate incubated at 37
After incubation at 37°C and 0% CO₂ for 1 hour. For the HCT116 cells, port A of the Sensor Cartridge was loaded with 30 µM oligomycin in Seahorse Assay Medium (from 5 mM oligomycin stock dissolved in DMSO), port B was loaded with 5 µM FCCP in Seahorse Assay Medium (from 5 mM FCCP stock dissolved in DMSO), and port C was loaded with 5 µM Rotenone/Antimycin A solution in Seahorse Assay Medium (from 10 mM rotenone stock dissolved in DMSO and 20 mM Antimycin A stock dissolved in DMSO), for final well concentrations of 3 µM oligomycin, 0.5 µM FCCP, and 0.5 µM Rotenone/Antimycin A. For the HIEC-6 cells, the final well concentrations were 3 µM oligomycin, 1 µM FCCP, and 0.5 µM Rotenone/Antimycin A. The Sensor Cartridge was placed in the Seahorse analyzer 15 minutes before the end of the Cell Culture Miniplate incubation time, and the Seahorse analyzer allowed to calibrate. The Miniplate was then also placed in the Seahorse analyzer and assay run.

The Cell Culture Miniplate was prepared for storage by removing the media from the wells of the Miniplate and rinsing the wells with 80 µL PBS. The PBS was removed, and the plate stored at -80°C. The amount of protein in each of the wells was determined by lysing the cells with 6.5 µL RIPA and doing BSA protein determination with a 10x dilution of the samples, and the amount of protein used to normalize the results if required. Three replicates were done, and the data analyzed with One-Way ANOVA and the significance determined with Dunnett’s test.

### 2.8 BrdU Treatment and Staining

HCT116 cells were plated in 6 cm plates at 375,000 cells/plate 48 hours before harvest. The cells were incubated at 37°C and 5% CO₂ for 24 hours, and then treated with nAg, AgNO₃ and sodium ascorbate for 24 hours. Control plates were done with no treatment and no stain, no
treatment and Propidium Iodide (PI), no treatment and 30 µM BrdU, no treatment with 30 µM BrdU and PI. One hour before the end of the treatment time the media was removed and replaced with 30 µM BrdU in media and incubated for 1 hour. The BrdU treatment was removed, the plates rinsed once with PBS, and the cells harvested with 1× trypsin. The trypsin was stopped with media, the cells transferred to Eppendorf tubes and centrifuged 1,000 ×g for 1 min, and the solution removed. The cell pellets were washed once with PBS and then fixed and resuspended in 1 mL ice-cold 70% ethanol. The cells were incubated at -20°C for a minimum of 30 min or up to two weeks. The ethanol was removed by centrifuged 1,000×g for 2 min and the cell pellets washed with PBS. The cell pellets were resuspended in 1 mL 50µg/mL RNase A in PBS and allowed to incubate at 37°C for 30 min. 0.4 mL PBS was added to the RNase A solution and the samples centrifuged 1,000×g for 2 min and the supernatant removed. The cells were resuspended in 1 mL ice-cold 0.1 N HCl with 0.7% Triton X-100 and incubated on ice for 15 min. After the incubation, 0.4 mL PBS was added to the solution and the samples were centrifuged at 1,000×g for 2 min and the supernatant removed. The cell pellets were resuspended in 0.7 mL sterile ddH₂O and incubated at 97°C on a hot block (Isotemp, Fisher Scientific, Waltham, Massachusetts, USA) for 15 min and then immediately cooled on ice for 15 min. After this incubation, 0.7 mL PBS with 0.5% Tween 20 was added to the cell solution and then centrifuged at 1,000×g for 2 min and the supernatant removed. The cells were resuspended in 50 µL of 1 in 100 diluted BrdU Monoclonal Antibody (Thermo Fisher, Invitrogen BrdU Monoclonal Antibody [MoBU-1], Alexa Fluor 488, Catalog# B35130), and incubated in the dark at room temperature for 30 min. After this incubation, 1 mL freshly made HBT (5% FBS and 0.5% Tween 20 in PBS) was added and the samples centrifuged at 1,000 ×g for 2 min and the supernatant removed. The cell pellets were washed in 150 µL HBT and then resuspended in 200 µL PBS with 40 µg/mL
RNase A and 20 μg/mL PI and incubated at 4°C for 30 min or overnight before being analyzed on the flow cytometer (BD Accuri C6 flow cytometer).

Cell debris and dead cells were removed from analysis by plotting FSC-A vs SSC-A and gating around the live cell population, and cell doublets were removed from the analysis of the live cells by plotting FSC-A vs FSC-H and selecting the singlets. Cell cycle singlets were further selected for by plotting FL2-A vs SSC-A and selecting the singlet cell population. The G1, S, and G2/M cell populations were found by plotting FL2-A with a linear scale on the x-axis vs FL1-A with a log scale on the y-axis. Colour compensation was done for all the samples and FL1 was corrected by subtracting 1.4% of FL2. The counts of the cell populations in the G1, S, and G2/M phases were added together and the proportion of cells in the individual cell cycle phases were determined relative to this. A minimum of three replicates were done, and the data analyzed with One-Way ANOVA and the significance determined with Dunnett’s test.

2.9 Flow Cytometry Analysis of stably transfected HCT116-ATF4 and HCT116-XBP1 cells

HCT116-ATF4 and HCT116-XBP1 stably transfected cells were plated in a 24 well plate at 4.0 × 10^5 cells/mL in DMEM media with 5% FBS and 5% NCS. One well of HCT116 cells were plated along with the stably transfected cells. The cells were incubated at 37°C and 5% CO₂ for 24 hours, and then treated with nAg, AgNO₃, and 200 nM thapsigargin and incubated for 24 hours in either a hypoxic (1% O₂) or normoxic (~21% O₂) environment in a ThermoForma Series II Water Jacketed CO₂ Incubator. The wells were gently washed three times with sterile PBS, harvested with 1× trypsin, and the trypsin stopped with media. The media was removed by centrifugation at 1,000 ×g for 1 min, the cell pellet rinsed with PBS, and then resuspended in
flow buffer (0.5% BSA and 2 mM EDTA in PBS). Cell samples were kept on ice and analyzed with flow cytometry (BD Accuri C6 flow cytometer).

Cell debris and dead cells were removed from analysis by plotting FSC-A vs SSC-A and gating around the live cell population, and cell doublets were removed from the analysis of the live cells by plotting FSC-A vs FSC-H and selecting the singlets. A histogram of the FL2 channel (for the HCT116-ATF4 cells) or FL1 channel (for the HCT116-XBP1 cells) vs count was done to find the mean fluorescence. The mean fluorescence of HCT116 cells was subtracted from the mean fluorescence of the stably transfected cells, and the data was normalized to the fluorescence of the untreated HCT116-ATF4 or HCT116-XBP1 cells. A minimum of three replicates was done, and the data analyzed with One-Way ANOVA and the significance determined with Dunnett’s test.

2.10 Luciferase

HEK 293T cells were plated in 60 mm cell culture dishes at 1.1 × 10^6 cells/plate and incubated at 37°C and 5% CO₂ for 24 hours. The cells were transfected with PEI (1 mg/mL working PEI solution) with 9 µg luciferase plasmid, 1 µg overexpressed plasmid or an empty plasmid, and 0.4 µg β-galactosidase plasmid (to correct for transfection efficiency). One plate was left untransfected to account for natural β-galactosidase levels. After 24 hours, the transfection media was removed, and the cells were treated for 24 hours. The media was then removed, and the cell plates rinsed twice with PBS. The cells were scraped into PBS, and the PBS removed after centrifugation at 650×g for 8 min (Sorvall Legend Micro 21 Centrifuge, Thermo Scientific, Waltham, Massachusetts). Luciferase Buffer (25 mM glycylglycine, 15 mM KH₂PO₄, 15 mM MgSO₄, and 4 mM EDTA; pH 7.8) was made fresh each day. The cell pellet
was lysed with 50 µL Luciferase Lysis Buffer (1 mM DTT, 1% Triton X-100, and 1x Protease Inhibitor added to the needed volume of Luciferase Buffer). The lysates were incubated on a rotator at 4°C for 20 – 30 min and then centrifuged at 13,000 ×g or higher for 15 – 30 min at 4°C. For the luciferase reading, 10 µL cell lysate was put in a 96 well white plate in triplicate and 76 µL Luciferase Assay Buffer (2 mM DTT and 2 mM ATP added to the needed volume of Luciferase Buffer) added to each of the wells. The plate was placed in a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenberg, Germany) and 50 µL Luciferin Solution (2 mM DTT and 200 µM Luciferin added to the needed volume of Luciferase Buffer) injected into each well and the luminescence read. Beta-galactosidase Buffer (60 mM Na₂HPO₄, 10 mM KCl, and 1 mM MgCl₂; pH 8.0) was made fresh each day. For the β-galactosidase assay, 50 µL of the luciferase lysate from each well of the white plate after the luminescence had been read was transferred to a 96 well clear bottom plate and 85 µL β-galactosidase Assay Buffer (1 mM DTT and 0.2 mg/mL CPRG added to the needed volume of β-galactosidase Buffer) added. The plate was incubated at 37°C for about 30 min or until the colour had developed, and the absorbance was read at 570 nm with a background subtraction at 630 nm (PowerWave XS microplate reader, BioTek Instruments, Winooski, Vermont).

The luminescence readings for the untrasfected sample were averaged and subtracted from the luminescence readings of the samples. The β-galactosidase absorbance readings for the untrasfected sample were averaged and subtracted from the β-galactosidase absorbance readings of the samples. The luminescence values of the samples were divided by their β-galactosidase values, and the data normalized to the transfected untreated sample.

The ARE-luciferase reporter plasmid pGL4.37[luc2P/ARE/Hygro] Vector (Promega, Madison, WI, USA) was used, containing four copies of the antioxidant response element (ARE)
from the GST Ya gene (Dhakshinamoorthy & Jaiswal, 2002) in the luciferase reporter gene 
\textit{luc2P} (\textit{Photinus pyralis}). The xenobiotic response element (XRE) luciferase reporter p1A1-FL was used, with the 5’ region of the human CYP1A1 gene from -1566 to +73 cloned into the pGL3-Basic vector (Promega) upstream of the firefly luciferase reporter gene (Morel & Barouki, 1998), and was a kind gift from Dr. Robert Barouki (Centre Universitaire des Saints-Pères, Saints-Pères, Paris, France). The XRE luciferase reporter pGudLuc7.5 was used, containing 20 dioxin-responsive elements (DRE) from the CYP1A1 promoter region oriented in the forward direction upstream of the mouse mammary tumor virus promoter of the pGudLuc1.0 luciferase reporter plasmid, and was a kind gift from Dr. Michael Denison (University of California, Davis, California, USA) (He et al., 2011). The C-FLAG tagged human aryl hydrocarbon receptor (AhR) open reading frame mammalian expression plasmid (AhR-CF plasmid, catalog number HG10454-CF) in a pCMV3-C-FLAG mammalian expression vector with a cytomegalovirus (CMV) promotor was obtained from Sino Biological Inc. (Wayne, PA, USA). The Nrf2 plasmid pCDNA3-myc3-Nrf2 in a mammalian expression vector with a CMV promotor was used (Addgene 21555). The N-Flag Nrf1 plasmid had the human Nrf1 gene cloned into pCR3.1 with an N-terminal FLAG tag, and the CMV-Nrf1 plasmid had the Nrf1 gene cloned into the CMV-5a-FLAG expression vector with a C-terminal FLAG tag (Chepelev et al., 2011). pCR3.1 was used for the empty plasmid control (Sigma-Aldrich, St. Louis, MO, USA). The β-galactosidase plasmid was used for a transfection control and was a kind gift from Dr. Henry Jay Forman (University of California, Davis School of Medicine, California, USA).
2.11 Western Blotting

2.11.1 Cell Plating and Treatment

HCT116 or HEK293T cells were plated in 10 cm plates at $1.5 \times 10^6$ cells/plate 48 hours before harvest ($7.5 \times 10^5$ cells/plate 72 hour before harvest). The cells were treated 4 hours before harvest for HCT116 cells and 2 hours before harvest for HEK293T cells. Co-treatment with 5 mM NAC or 10 µM buthionine sulfoximine (BSO) was done in conjunction with nanosilver treatment for 4 hours prior to harvest. Twenty µM TBHQ (from 100 mM stock dissolved in ethanol diluted to 10 mM stock in media), 5 µM SFN (from 25 mg/mL, or 141,004 µM stock, dissolved in ethanol), and 7.5 nM TCDD (from 10,000 nM stock dissolved in DMSO) were used as positive controls.

2.11.2 Nuclear and Cytosolic Fractionation

The plates were first rinsed 3 times with 1× PBS and then scraped into 750 µL 1× PBS. The cells were centrifuged at 680×g for 10 min and the supernatant discarded. The cell pellet was flash frozen in liquid nitrogen and stored at -80°C until needed. The cytosolic fraction was obtained by resuspending the cell pellets in 3.5× the cell pellet volume equivalent (3.5 mL of Buffer A for every gram of cell pellet) of Buffer A (10 mM KCl, 10 mM HEPES, 1.5 mM MgCl₂, 1 mM DTT, 0.5 mM PMSF, and 1× protease inhibitor, pH 7.9), and incubating the samples on the rotator at 4°C for 15 min. 1% NP-40 was added to the cell suspensions for a final concentration of 0.1% NP-40 (v/v), and the tubes carefully flicked by hand 13-15 times. The samples were centrifuged at 680×g for 10 min (this centrifugation was repeated if needed to keep the pellet at the bottom while removing the supernatant) and the supernatant collected as the cytosolic fraction. The pellets were washed twice with 150 µL Buffer A. The pellets were
resuspended in 40 µL RIPA buffer (with 1 mM DTT, 0.5 mM PMSF, and 1× protease inhibitor added). The cell pellets were vigorously mixed by running the tubes 10 – 15× along a tube rack. The samples were then incubated on a rotator at 4°C for 30 min. The samples were centrifuged at maximum speed (at least 14,000×g) for 30 min at 4°C. The supernatant was transferred to clean tubes as the nuclear fraction and the pellets discarded, and the samples stored at -80°C until needed.

2.11.3 Cell Lysis and Protein Extraction using Urea

The media was removed from the plates and the plates rinsed three times with PBS. If the plates had been in hypoxic treatment, the plates were rinsed with PBS that had been bubbled with nitrogen prior to use. The cells were scraped into 1 mL PBS and centrifuged at 1000×g for 1 min and the supernatant discarded. The cell pellets were washed in PBS, the PBS removed, and the cell pellets lysed with 100 µL 8M urea dissolved in ddH₂O. For hypoxic treated samples the PBS wash was omitted to decrease the time before lysis. The samples were rotated at 4°C overnight, and then centrifuged at 100,000×g for 1 hour at 4°C using an ultracentrifuge (Sorvall RC-M150GX, Thermo Scientific, Waltham, Massachusetts). The samples were stored at -80°C until needed.

2.11.4 Total Protein Determination and SDS-PAGE Gel Electrophoresis

The total protein concentration of the samples was determined using the bicinchoninic acid (BCA) assay with a 10x dilution of the samples (Pierce™, ThermoFisher Scientific, Ottawa, ON, Canada).
For the cytosol and nuclear fraction samples 15 µg of protein was used while for the urea extracted samples 30 µg of protein was used. The samples were diluted with the appropriate buffer and added to 2× Laemmlli sample buffer in a 1:1 ratio (Bio-Rad, Hercules, CA, USA). The samples were heated at 100°C for 5 min, loaded onto an 8% (for AhR blotting) or 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and run at 150 V in Resolving Buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3); including one lane for the Precision Plus Protein All Blue Standards 10 – 250 kDa protein ladder (Bio-Rad, Hercules, CA, USA). 0.5% TCE was included when making the SDS-PAGE gel and the image after photoactivation of the gel with UV light was used for total protein normalization (Ladner et al., 2004). The protein was transferred from the gel to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) overnight at 4°C and 180 mA in Transfer Buffer (20 mM Tris, 150 mM glycine, and 20% methanol, pH 8.0). The membrane was blocked using 10% w/v nonfat dry milk in TBST (20 mM Tris, 137 mM NaCl, and 0.1% Tween 20, pH 7.6) for 1 hour at 4°C.

2.11.5 Antibodies

The membranes with the cell fractionation samples were always probed with Lamin B and β-tubulin to check for proper nuclear and cytosolic separation, respectively. The membrane was incubated with primary antibody overnight at 4°C, washed in TBST for 5 min five times, and then incubated with the secondary antibody for 1 hour at 4°C. The antibodies used and their dilutions are listed in Table 1. Clarity Western ECL blotting substrate (Bio-Rad, Hercules, California) was used for visualization.

The Western blot images were analyzed using Image Lab 6.1 Software (Bio-Rad, Hercules, Massachusetts) to determine the densitometry of the bands and total protein
normalization was done using the TCE image. Normalized data was statistically tested for significance using One-Way ANOVA and the Dunnett test.

**Table 1:** List of antibodies, the dilutions used, and the observed molecular weights.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Catalog Number</th>
<th>Type</th>
<th>Dilution</th>
<th>Observed Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>Ab 62352*</td>
<td>Rabbit monoclonal</td>
<td>1:5000 in TBST with 5% w/v BSA and 0.01% Sodium Azide</td>
<td>100 kDa</td>
</tr>
<tr>
<td>Nrf1</td>
<td>NBP2-55915**</td>
<td>Rabbit polyclonal</td>
<td>1:1000 in TBST with 5% w/v BSA and 0.01% Sodium Azide</td>
<td>95 kDa</td>
</tr>
<tr>
<td>AhR</td>
<td>Invitrogen JM34-10***</td>
<td>Rabbit monoclonal</td>
<td>1:1000 in TBST with 5% w/v BSA and 0.01% Sodium Azide</td>
<td>105 kDa</td>
</tr>
<tr>
<td>Lamin B</td>
<td>sc M-20****</td>
<td>Goat polyclonal</td>
<td>1:1000 in TBST with 5% w/v BSA and 0.01% Sodium Azide</td>
<td>67 kDa</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Developmental Studies Hybridoma Bank (DSHB) product E7</td>
<td>Mouse monoclonal</td>
<td>1:1000 in TBST with 5% w/v BSA and 0.01% Sodium Azide</td>
<td>50 kDa</td>
</tr>
<tr>
<td>Hif-1α</td>
<td>D2U3T*****</td>
<td>Rabbit monoclonal</td>
<td>1:1000 in TBST with 5% w/v BSA and 0.01% Sodium Azide</td>
<td>120 kDa</td>
</tr>
<tr>
<td>Anti-Wntless</td>
<td>655902******</td>
<td>Mouse monoclonal</td>
<td>1:1000 in TBST with 5% w/v milk and 0.01% Sodium Azide</td>
<td>41 kDa</td>
</tr>
<tr>
<td>Wnt-3a</td>
<td>NBP1-19050**</td>
<td>Mouse monoclonal</td>
<td>1:1000 in TBST with 1% w/v milk and 0.01% Sodium Azide</td>
<td>39 kDa</td>
</tr>
<tr>
<td>Wnt-5a</td>
<td>NBP2-24752**</td>
<td>Rabbit polyclonal</td>
<td>1:1000 in TBST with 1% w/v milk and 0.01% Sodium Azide</td>
<td>35 kDa</td>
</tr>
<tr>
<td>PORCN</td>
<td>NBP1-59677**</td>
<td>Rabbit polyclonal</td>
<td>1:1000 in TBST with 0.01% Sodium Azide</td>
<td>63 kDa</td>
</tr>
<tr>
<td>β-catenin</td>
<td>NBP1-54467**</td>
<td>Mouse monoclonal</td>
<td>1:1000 in TBST with 0.01% Sodium Azide</td>
<td>100 kDa</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td>Catalog Number</td>
<td>Type</td>
<td>Dilution</td>
<td>Observed Molecular Weight</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------------------</td>
<td>------------</td>
<td>-------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Goat Anti-Rabbit</td>
<td>Ab 6721*</td>
<td></td>
<td>1:10,000 in TBST</td>
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<tr>
<td>Goat Anti-Mouse</td>
<td>Dako P0447</td>
<td></td>
<td>1:5000 in TBST</td>
<td></td>
</tr>
<tr>
<td>Mouse Anti-Goat</td>
<td>sc-2354****</td>
<td></td>
<td>1:2000 in TBST</td>
<td></td>
</tr>
</tbody>
</table>

* Abcam, Cambridge, Massachusetts, USA
** Novus Biologicals, Oakville, Ontario, Canada
*** Invitrogen, Thermo Fisher Scientific, Waltham, MT, USA
**** Santa Cruz Biotechnology Inc., Dallas, Texas, USA
***** Cell Signaling Technology, Danvers, MA, USA
****** BioLegend, San Diego, CA, USA

2.12 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

HCT116 cells were plated at $6 \times 10^5$ cells/plate 48 hours before harvest and treated 4 hours before harvest. The plates were quickly rinsed with 37°C PBS and the cells immediately lysed on the plate. Total ribonucleic acid (RNA) was extracted according to the Aurum Total RNA Mini Kit (BioRad, Hercules, California, USA), and the quality of the RNA analyzed by nanodrop and visualization on a bleached agarose gel (1% agarose gel with 1% v/v bleach). One microgram of RNA was converted to complementary deoxyribonucleic acid (cDNA) using the iScript cDNA Synthesis Kit (BioRad, Hercules, California, USA). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using Sso-Advanced Universal SYBR Green (BioRad, Hercules, California, USA) at 60°C, and analyzed using CFX Manager (BioRad software). The primers were validated with a thermal gradient, an agarose gel of the amplicons obtained from the thermal gradient, and standard curves. The specificity of the primers was checked by including a melt curve for every plate to see that there were no primer dimers. A non-template control with DNase/RNase free water was included for each primer pair on each plate. The primer sequences and the quantity of cDNA used for each primer pair are
listed in Table 2. Three independent experiments were done, and the data analyzed with One-Way ANOVA and the significance determined with Dunnett’s test.

Table 2: Primer sequences and the amount of cDNA used in the qRT-PCR reaction for each primer pair.

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Primer Sequence</th>
<th>Amount of cDNA per Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf 1</td>
<td>Forward: 5'-CTG GAG GAG GAA TTT GAC TCT G-3' Reverse: 5'-GAG GAA GAG GAG GAG GAA GAA-3'</td>
<td>500 ng</td>
</tr>
<tr>
<td>Nrf 2</td>
<td>Forward: 5'-CCG GCA TTT CAC TAA ACA CAA G-3' Reverse: 5'-CAG AAT CAC TGA GGC CAA GTA G-3'</td>
<td>250 ng</td>
</tr>
<tr>
<td>AhR</td>
<td>Forward: 5'-TCA ACA GCA ACA GTC CTT GG-3' Reverse: 5'-TCC AAT TTT CAA ACA TGC CA-3'</td>
<td>250 ng</td>
</tr>
<tr>
<td>WLS</td>
<td>Forward: 5'-GGA CAT TGC CTT CAA GCT AAA C-3' Reverse: 5'-CAT TTC AGT CCA CTC AGC AAA C-3'</td>
<td>500 ng</td>
</tr>
<tr>
<td>WNT3A</td>
<td>Forward: 5'-GAC TTC CTC AAG GAC AAG TAC G-3' Reverse: 5'-GGC ACC TTG AAG TAG GTG TAG-3'</td>
<td>1000 ng</td>
</tr>
<tr>
<td>WNT5A</td>
<td>Forward: 5'-CCC AGG ACC CGC TTA TTT ATA G-3' Reverse: 5'-GGT TCC GGT TGC AAT TCT TG-3'</td>
<td>1000 ng</td>
</tr>
<tr>
<td>PORCN</td>
<td>Forward: 5'-CTC CTT CCA CTT CAG CAA CTA T-3' Reverse: 5'-CCA TTC CAG GTG ATC CTT CTC-3'</td>
<td>1000 ng</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-CTT TGG TAT CGT GGA AGG ACT C-3' Reverse: 5'-GAG GCA GGG ATG ATG TTC TG-3'</td>
<td>10 ng</td>
</tr>
</tbody>
</table>
Chapter 3: Results

3.1 Nanosilver Characterization

Particle size, shape, surface coating, surface charge, protein corona formation all have a significant effect on the biological effects on cells. Smaller particles can more easily enter the cells and interact with the cellular components, while larger particles mainly remain outside the cells and interact with the cell membrane and receptors, or be taken in through phagocytosis (Hewitt et al., 2020). Aggregation of the particles results in a larger overall particle size. Additionally, protein coronas can form on the surface of the nanoparticles, increasing their overall size. Hard coronas form if the proteins strongly bind to the surface, and soft coronas from if the proteins bind weakly to the surface. A soft corona may form around the nanoparticle itself or on top of a hard corona. These protein coronas may alter how the cell interacts with the nanoparticle, especially for larger nanoparticles which may absorb a large amount of protein (Hewitt et al., 2020; Liu et al., 2017; Mariam et al., 2016).

In this study, 10 nm nAg was used. This size is close to the size of biological proteins and DNA, and the nAg was observed to be inside the treated cells at the higher treatment concentrations (see Figure 4). Polyvinylpyrrolidone and citrate were chosen for the surface coatings in this study since these are commonly used surface coatings to stabilize the nAg, and do not appear to cause the biological effects in cells that are observed with the coated nAg (Armstrong et al., 2013; Das et al., 2014; Lin et al., 2018; Wu et al., 2015, 2016). In this study, some initial cell viability studies were done using 10 nm citrate coated nAg (citrate-nAg), however, due to stability issues, the experiments were done using 10 nm PVP-coated nAg (PVP-nAg).
**Figure 4:** HIEC cells treated with 25 µg/ml PVP-nAg for 24 hours. Nanosilver is observed inside the cells. The magnification is 20×.

The 10 nm PVP-nAg was characterized by TEM and by measuring the UV-Vis spectrum to ensure its size and stability. The average particle size was 9.99 ± 0.37 nm, as measured by TEM, with 59.3% of the particles falling between 8 – 12 nm (see Figure 5). The particles were seen to be mainly single spheres (see Figure 6).
**Figure 5:** The TEM particle size distribution by relative percentage.
Figure 6: The TEM images of the 10 nm PVP-nAg at magnifications of: A) 100,000×, B) 50,000×, and C) 2,000,000×.
The UV-Vis spectrum showed a single large peak at 396 nm (see Figure 7A). This is within the acceptable range for 10 nm nAg of 380 – 405 nm, indicating that it is approximately the correct size, and the presence of only one peak indicates that the nAg is stable as single nanoparticles and has not aggregated. Destabilized nAg is shown in Figure 7B, and the decrease in the amount of 10 nm sized silver nanoparticles is indicated by the decrease in the primary absorbance peak at 388 nm. The presence of the secondary absorbance peak at the longer wavelength of 608 nm indicates the presence of larger sized aggregated particles. Nanosilver was only used in the experiments in this study when it was of good quality and not aggregated.
Figure 7: The UV-Vis Spectrum of 10 nm PVP-nAg measured in ddH₂O water. A) Good quality nAg with a peak absorbance of 0.932 at 396 nm, and B) aggregated nAg with two peaks with absorbances of 0.470 at 388 nm and 0.245 at 608 nm.
3.2 Determining the AgNO₃ Control Treatment Concentrations

The standard curve for the measured silver ion concentration from solutions of 0, 0.05, 0.1, 1, 2, and 5 µg/mL AgNO₃ incubated in cell culture media for 24 hours is shown in Figure 8A. The silver ion concentrations were measured by ICP-MS. The equation of the line of the silver nitrate standard curve \( y = 644.41x - 57.72 \), where \( x \) is the AgNO₃ concentration, and \( y \) is the silver ion concentration) was used to determine the equivalent concentrations of AgNO₃ for 1, 5, 10 µg/mL PVP-nAg from the measured level of silver ions released at 1, 7, and 25 hours (see Figure 8B).

The equivalent AgNO₃ concentrations at 4-hours was calculated from the equations of the curves in Figure 8B for 1, 5, and 10 µg/mL PVP-nAg and graphed in Figure 8C. The equation of the resulting curve \( y = 0.5808x^{0.3487} \), where \( x \) is the nAg concentration, and \( y \) is the equivalent AgNO₃ concentration at 4 hours) was then used to calculate AgNO₃ control concentrations for 4-hour PVP-nAg treatment. The results of the calculations are shown in Table 3.

The estimated AgNO₃ control concentrations for 4-hour PVP-nAg treatment for various concentrations are shown in Table 3. The control values used in this study for the 4-hour treatment are: 0.59 µg/mL AgNO₃ to correspond to 1 µg/mL PVP-nAg, 1.33 µg/mL AgNO₃ to correspond to 10 µg/mL PVP-nAg, 1.78 µg/mL AgNO₃ to correspond to 20 µg/mL PVP-nAg, and 1.90 µg/mL AgNO₃ to correspond to 30 µg/mL PVP-nAg. The AgNO₃ control concentration for the Cell Mito Stress Test Seahorse experiment was 1.65 µg/mL AgNO₃ to correspond to 20 µg/mL PVP-nAg.

The estimated AgNO₃ control concentrations chosen in this study for 24-hour treatment were chosen using the EC₅₀ value of the cells (see Table 4) to allow for cell viability in the
experiments, and were 1, 1.5, and 2 µg/mL AgNO₃ to correspond to 1, 10, and 20 µg/mL PVP-nAg respectively.

![Graph A](image)

**Equation A:**

\[ y = 644.41x - 57.72 \]

\[ R^2 = 0.9956 \]

![Graph B](image)

**Equation B:**

\[ y = -0.0030x^2 + 0.1607x + 0.7309 \]

\[ R^2 = 1 \]

\[ y = -0.0010x^2 + 0.0549x + 0.7821 \]

\[ R^2 = 1 \]

\[ y = -0.0015x^2 + 0.0611x + 0.3673 \]

\[ R^2 = 1 \]
Figure 8: Silver ions released from AgNO₃ and PVP-nAg in media as measured by ICP-MS, and calculation of the equivalent AgNO₃ concentrations for PVP-nAg after incubation for 4 hours. A) The standard curve of the measured silver ion concentration from solutions of 0, 0.05, 0.1, 1, 2, and 5 µg/mL AgNO₃. B) The equivalent AgNO₃ concentrations determined from the standard curve using the measured concentration of silver ions released from 1, 5, 10 µg/mL nAg after incubation for 1, 7, and 25 hours. C) The equivalent AgNO₃ concentrations at 4 hours determined by the AgNO₃ concentrations calculated for 1, 5, and 10 µg/mL nAg from the equations in B. The data is from one experiment.
Table 3: Calculated AgNO₃ concentrations for 10 nm PVP-nAg incubated for 4 hours.

<table>
<thead>
<tr>
<th>4-hour Incubation Time</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated AgNO₃ equivalent for 1 µg/mL nAg (from ( y = -0.0015x^2 + 0.0611x + 0.3673 ))</td>
<td>0.59 µg/mL</td>
</tr>
<tr>
<td>Calculated AgNO₃ equivalent for 10 µg/mL nAg (from ( y = -0.0030x^2 + 0.1607x + 0.7309 ))</td>
<td>1.33 µg/mL</td>
</tr>
<tr>
<td>Percent increase of the AgNO₃ equivalent from 5 to 10 µg/mL nAg</td>
<td>134.49 %</td>
</tr>
<tr>
<td>Estimated AgNO₃ equivalent for 20 µg/mL nAg using the calculated AgNO₃ equivalent for 10 µg/mL nAg and the percent increase of the AgNO₃ equivalent from 5 to 10 µg/mL nAg</td>
<td>1.78 µg/mL</td>
</tr>
<tr>
<td>Calculated AgNO₃ equivalent for 20 µg/mL nAg at 4 hours (from ( y = 0.5808x^{0.3487} ))</td>
<td>1.65 µg/mL</td>
</tr>
<tr>
<td>Calculated AgNO₃ equivalent for 30 µg/mL nAg at 4 hours (from ( y = 0.5808x^{0.3487} ))</td>
<td>1.90 µg/mL</td>
</tr>
</tbody>
</table>

3.3 Cell Viability with nAg, AgNO₃, and SA Treatments

3.3.1 Cell Viability with nAg Treatments

The cell viability when treated with the PVP-nAg or citrate-nAg is shown in Figure 9. The PVP-nAg was found to be non-toxic to the non-cancerous HIEC-6 colon cells at treatment concentrations up to 125 µg/mL for 24 hours (Figure 9A and Table 4). In comparison to these cells, cytotoxicity was found with the PVP-nAg treatment of the HCT116 colon cancer cells, which had an EC₅₀ value of 78.43 ± 0.70 µg/mL when treated for 24 hours (see Figure 9B and
Table 4). Two-Way ANOVA analysis showed that there is a statistically significant decrease in viability of the HCT116 colon cancer cells treated with PVP-nAg as compared to the HIEC-6 cells (see Figure 10).

The HEK293T human embryonic kidney cells were found to be even more susceptible to the PVP-nAg treatment and had an EC$_{50}$ value of 48.24 ± 1.86 µg/mL when treated for 24 hours (see Figure 9C and Table 4). A shorter treatment time of 6 hours by the PVP-nAg resulted in less toxicity than the longer time treatment and had an EC$_{50}$ value of 65.06 ± 4.73 µg/mL in the HEK293T cells (see Figure 9C and Table 4). Not all concentrations were tested for this treatment time since there were no changes in the 24-hour treatment times at the lower concentrations, so they were excluded in the 6-hour treatments.

The citrate-nAg was found to be much more toxic to the HEK293T cells than the PVP-nAg and had an EC$_{50}$ value of 6.95 ± 0.02 µg/mL for 24 hour treatment (see Figure 9D and Table 4). Neuroblastoma SH-SY5Y cells and mouse macrophage RAW264.7 cells experienced less toxicity than the HEK293T cells, with 24 hour treatment EC$_{50}$ values of 23.61 ± 0.01 µg/mL and 42.60 ± 0.01 µg/mL respectively (see Figure 9E, Figure 9F, and Table 4).
**Figure 9:** The cell viability of HIEC-6, HCT116, HEK293T, SH-SY5Y, and RAW264.7 cells treated with 10 nm PVP-nAg or 10 nm citrate-nAg for 6 or 24 hours as measured by the MTT cell viability assay. A) HIEC-6 cells treated with PVP-nAg for 24 hours, B) HCT116 cells treated with PVP-nAg for 24 hours, C) HEK293T cells treated with PVP-nAg for 6 and 24 hours, D) HEK293T cells treated with citrate-nAg for 24 hours, E) SH-SY5Y cells treated with citrate-nAg for 24 hours, and F) RAW264.7 cells treated with citrate-nAg for 24 hours. The data is the mean of 3 to 12 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 10: The cell viability of HIEC-6 and HCT116 cells treated with 10 nm PVP-nAg for 24 hours as measured by the MTT cell viability assay. The data is the mean of 4 independent experiments ± S.E.M. Statistical analysis was done using Two-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.

3.3.2 Cell Viability with AgNO₃ Treatments

The cell viability when treated with AgNO₃ for 6 or 24 hours is shown in Figure 11. The AgNO₃ was least toxic to the non-cancerous HIEC-6 colon cells with an EC₅₀ value of 17.97 ± 0.36 µg/mL when treated for 24 hours (Figure 11A and Table 4). In fact, the highest AgNO₃ treatment concentration had to be doubled from what the other cell lines were treated with in order to observe a significant effect. The AgNO₃ was found to be very toxic to the HCT116 cells with an EC₅₀ value of 1.64 ± 0.03 µg/mL when treated for 24-hours (see Figure 11B and Table 4). Two-Way ANOVA analysis showed that there is a statistically significant decrease in
viability of the HCT116 colon cancer cells treated with AgNO₃ as compared to the HIEC-6 cells (see Figure 12).

Twenty-four hour treatment of HEK293T cells with AgNO₃ was also found to be very toxic with an EC₅₀ value of 1.44 ± 0.25 µg/mL (see Figure 11C and Table 4). As expected, a shorter 6-hour treatment of the HEK293T cells resulted in reduced toxicity with an EC₅₀ value of 2.63 ± 0.14 µg/mL (see Figure 11C and Table 4). Not all concentrations were tested for this treatment time since there were no changes in the 24-hour treatment times at the lower concentrations, so they were excluded in the 6-hour treatments.

The cell viability of the SH-SY5Y and RAW264.7 cells was slightly more than that of the HEK293T and HCT116 cells, but was still below that of the HIEC-6 cells, with an EC₅₀ value of 4.00 ± 0.04 µg/mL for the SH-SY5Y cells and 3.57 ± 0.02 µg/mL for the RAW264.7 cells (see Figure 11D, Figure 11E, and Table 4).
<table>
<thead>
<tr>
<th>AgNO₃ Concentration (µg/mL)</th>
<th>Absorbance (570 nm)</th>
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</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>0.10</td>
<td>0.4</td>
</tr>
<tr>
<td>0.50</td>
<td>0.6</td>
</tr>
<tr>
<td>1.00</td>
<td>0.8</td>
</tr>
<tr>
<td>5.00</td>
<td>1.0</td>
</tr>
<tr>
<td>10.00</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Figure 11:** The cell viability of HIEC-6, HCT116, HEK293T, SH-SY5Y, and RAW264.7 cells treated with AgNO₃ for 24 or 6 hours as measured by the MTT cell viability assay. A) HIEC-6 cells treated with AgNO₃ for 24 hours, B) HCT116 cells treated with AgNO₃ for 24 hours, C) HEK293T cells treated with AgNO₃ for 6 and 24 hours, D) SH-SY5Y cells treated with AgNO₃ for 24 hours, and E) RAW264.7 cells treated with AgNO₃ for 24 hours. The data is the mean of 3 to 13 individual experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 12: The cell viability of HIEC-6 and HCT116 cells treated with AgNO₃ for 24 hours as measured by the MTT cell viability assay. The data is the mean of 5–7 independent experiments ± S.E.M. Statistical analysis was done using Two-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.3.3 Cell Viability with SA Treatments, and nAg and SA Combined Treatments

When treated with sodium ascorbate for 24 hours, the HIEC-6 cells had an EC$_{50}$ value of $3.77 \pm 0.01$ mM, which is significantly lower than the HCT116 cells which had an EC$_{50}$ value of $5.37 \pm 0.92$ mM (see Figure 13A, Figure 13B, Figure 14, and Table 4). The viability of the HCT116 cells treated for 1 hour and then allowed to recover was greater than that observed with 24-hour treatment, and had an EC$_{50}$ value of $9.85 \pm 0.05$ mM (see Figure 13C and Table 4). It would be expected that the viability of the HIEC-6 cells would be higher as well if they were only treated for 1 hour and allowed to recover.

Combining nAg with sodium ascorbate treatments resulted in higher toxicity in the HCT116 cells as compared to the HIEC-6 cells. When low-dose 10 µg/mL PVP-nAg treatment was added to increasing concentrations of sodium ascorbate, the EC$_{50}$ value for the HCT116 cells greatly decreased from $5.37 \pm 0.92$ mM sodium ascorbate to $0.73 \pm 0.20$ mM sodium ascorbate (see Figure 13D and Table 4). The HIEC-6 cells experienced less of a decrease in cell viability when the nAg was added into the sodium ascorbate treatments even though they started with a lower EC$_{50}$ value, and went from an EC$_{50}$ value of $3.77 \pm 0.01$ mM sodium ascorbate to $1.71 \pm 0.05$ mM sodium ascorbate with the combined treatment. The combined treatment of constant 10 µg/mL PVP-nAg with increasing concentrations of sodium ascorbate resulted in a statistically significant decrease in HCT116 cell viability as compared to the HIEC-6 cells by Two-Way ANOVA analysis (see Figure 15).

Incorporating a constant 1 mM sodium ascorbate treatment with the varying nAg treatments resulted in increased toxicity of the HCT116 cells while no decrease in viability was observed in the HIEC-6 cells (see Figure 13E). Although this combined treatment decreased the viability of the HCT116 cells, the EC$_{50}$ value was not found since it was higher than 100.00
µg/mL nAg with the added 1 mM sodium ascorbate (see Figure 13E and Table 4), and this decrease in HCT116 cell viability was not significantly different from the viability of the HIEC-6 cells by Two-Way ANOVA analysis. In fact, the EC\textsubscript{50} value of the HCT116 cells was lower when treated with just the nAg alone with an EC\textsubscript{50} value of 78.43 ± 0.70 µg/mL nAg. Thus, the low sodium ascorbate supplementation decreased the toxicity of the combined nAg treatment. Thus, in terms of combined treatments, it is much more advantageous to use a higher sodium ascorbate treatment level with low nAg treatment than to use low sodium ascorbate with high nAg treatment. This is a novel result that has not been reported before in the literature.
**A**

Sodium Ascorbate Concentration (mM)

![Graph showing absorbance (570 nm) against sodium ascorbate concentration.](image)

**B**

Sodium Ascorbate Concentration (mM)

![Graph showing absorbance (570 nm) against sodium ascorbate concentration.](image)
Figure 13: Effect of sodium ascorbate (SA) treatment and SA combined with PVP-nAg treatment on the viability of HIEC-6 and HCT116 cells as measured by the MTT cell viability assay. A) HIEC-6 cells treated with SA for 24 hours, B) HCT116 cells treated with SA for 24 hours, C) HCT116 cells treated with SA for 1 hour and then allowed to recover for 24 hours, D) HIEC-6 and HCT116 cells treated with 10 µg/mL PVP-nAg and varying concentrations of SA for 24 hours, and E) HIEC-6 and HCT116 cells treated with 1 mM SA and varying concentrations of PVP-nAg for 24 hours. The data is the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 14: The cell viability of HIEC-6 and HCT116 cells treated with Sodium Ascorbate for 24 hours as measured by the MTT cell viability assay. The data is the mean of 3 – 6 independent experiments ± S.E.M. Statistical analysis was done using Two-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 15: The cell viability of HIEC-6 and HCT116 cells treated with 10 µg/mL PVP-nAg and 0 – 10 mM Sodium Ascorbate (SA) for 24 hours as measured by the MTT cell viability assay. The data is the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using Two-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
**Table 4:** Nanosilver, AgNO₃, SA, and combined nAg and SA treatment EC₅₀ values for HIEC-6, HCT116, HEK293T, SH-SY5Y, and RAW264.7 cells.

<table>
<thead>
<tr>
<th>Treatment EC₅₀ Values</th>
<th>HIEC-6</th>
<th>HCT116</th>
<th>HEK293T</th>
<th>SH-SY5Y</th>
<th>RAW264.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP-nAg (µg/mL)</td>
<td>&gt; 125.00</td>
<td>78.43 ± 0.70</td>
<td>48.24 ± 1.86</td>
<td></td>
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</tr>
<tr>
<td>24-hour EC₅₀</td>
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<td></td>
<td></td>
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<tr>
<td>PVP-nAg (µg/mL)</td>
<td></td>
<td></td>
<td>65.06 ± 4.73</td>
<td></td>
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<tr>
<td>6-hour EC₅₀</td>
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<tr>
<td>Citrate-nAg (µg/mL)</td>
<td></td>
<td></td>
<td>6.95 ± 0.02</td>
<td>23.61 ± 0.01</td>
<td>42.60 ± 0.01</td>
</tr>
<tr>
<td>24-hour EC₅₀</td>
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<td></td>
</tr>
<tr>
<td>AgNO₃ (µg/mL)</td>
<td>17.97 ± 0.36</td>
<td>1.64 ± 0.03</td>
<td>1.44 ± 0.25</td>
<td>4.00 ± 0.04</td>
<td>3.57 ± 0.02</td>
</tr>
<tr>
<td>24-hour EC₅₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgNO₃ (µg/mL)</td>
<td>2.63 ± 0.14</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6-hour EC₅₀</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SA (mM)</td>
<td>3.77 ± 0.01</td>
<td>5.37 ± 0.92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-hour EC₅₀</td>
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<tr>
<td>1-hour followed by 24-hour recovery EC₅₀</td>
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<td>9.85 ± 0.05</td>
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<tr>
<td>Combined Varying SA</td>
<td>1.71 ± 0.05</td>
<td>0.73 ± 0.20</td>
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<tr>
<td>With Constant 10 µg/mL nAg (mM)</td>
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<tr>
<td>24-hour EC₅₀</td>
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<tr>
<td>Combined Varying nAg</td>
<td>&gt; 100.00 µg/mL nAg with 1 mM SA</td>
<td>&gt; 100.00 µg/mL nAg with 1 mM SA</td>
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<tr>
<td>With Constant 1 mM SA (µg/mL)</td>
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<tr>
<td>24-hour EC₅₀</td>
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3.4 Cell Pictures

The HIEC-6 cells were seen to be visibly healthy in the 24-hour PVP-nAg treatments up to 30 µg/mL and the 24-hour AgNO$_3$ treatments up to 2 µg/mL (see Figure 16). These were the highest treatment concentrations used in the 4-hour experiments. On the other hand, the HCT116 cells were observed to be round, shriveled, and smaller in the 1.5 and 2 µg/mL AgNO$_3$ treatments, and slightly distressed in the 24-hour 30 µg/mL PVP-nAg treatments (see Figure 17). Both the HIEC-6 and the HCT116 cells have some nAg particles visible inside them at the 30 µg/mL PVP-nAg treatment level.

Figure 16: HIEC-6 cells treated with 10 nm PVP-nAg and AgNO$_3$ for 24 hours. A) 10× magnification, scale bar is 400 µm; and B) 20× magnification, scale bar is 200 µm.
**Figure 17:** HCT116 cells treated with 10 nm PVP-nAg and AgNO₃ for 24 hours. A) 10× magnification, scale bar is 400 µm; and B) 20× magnification, scale bar is 200 µm.
3.5 Cell Cycle Analysis

Cell cycle arrest in the G2/M phase occurred in HCT116 cells treated for 24 hours with PVP-nAg (see Figure 18). Sodium ascorbate treatment at 1 mM did not induce cell cycle arrest in the HCT116 cells on its own; however, when it was combined with the 10 and 20 µg/mL PVP-nAg, increased cell cycle arrest in the G2/M phase was observed. This cell cycle arrest with the combined treatment was slightly more than that observed with the nAg treatment alone, although there was no significant difference between these. Interestingly, the AgNO₃ did not induce cell cycle arrest even at the highest treatment concentration above the EC₅₀ value (see Figure 18). This indicates that the cell cycle arrest in the G2/M phase observed with the nAg treatment is a silver nanoparticle-specific effect and is not the result of released silver ions or oxidative stress caused by the released silver ions.
Figure 18: Cell cycle analysis of the percentage of cells in the G1, S, and G2/M phases for HCT116 cells treated for 24 hours determined by BrdU incorporation and PI staining. A) Representative gating of the control samples, B) Two-parameter flow cytometric analysis for DNA content (FL2-A channel) vs BrdU incorporation (FL1-A channel) for a representative of each treatment, C) Cell cycle distribution for HCT116 cells treated with PVP-nAg, AgNO₃, SA, or SA with PVP-nAg for 24 hours. The data is the average of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.6 Cellular Oxidative Stress

Factors such as dose, treatment time, size of nAg used, and the sensitivity of the cells affect the amount of ROS produced by nAg. The level of cellular and mitochondrial oxidative stress produced by nAg in the HIEC-6 and HCT116 cells was measured in order to determine whether the non-toxic nAg treatments used in this work caused oxidative stress.

In this study, cellular ROS was measured using CellROX Deep Red. Cellular ROS was not produced in the HIEC-6 cells after 24-hour nAg and AgNO₃ treatment in either normoxic or hypoxic conditions (see Figure 19). Additionally, cellular ROS was not produced in the HCT116 cells after 24-hour nAg and AgNO₃ treatment in either normoxic or hypoxic conditions (see Figure 20). A shorter treatment time of 4 hours was also tested with the HCT116 cells, with this also showing no change in cellular ROS (see Figure 20A). Since no change in cellular ROS was observed in the HCT116 cells after 4 hours of treatment, this time point was not tested in the HIEC-6 cells.
Figure 19: Measurement of cellular oxidative stress in HIEC-6 cells using CellROX after 24 hours of treatment with PVP-nAg or AgNO₃ in: (A) normoxic conditions, or (B) hypoxic conditions. The values are expressed relative to the untreated control and are the mean of 3 to 5 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
**Figure 20:** Measurement of cellular oxidative stress in HCT116 cells using CellROX after 4 or 24 hours of treatment with nAg or AgNO₃ in normoxic or hypoxic conditions. A) 4-hour treatment in normoxic conditions, B) 24-hour treatment in normoxic conditions, and C) 24-hour treatment in hypoxic conditions. The values are expressed relative to the untreated control and are the mean of 3 to 5 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.7 Mitochondrial Oxidative Stress and Function

To determine whether the non-toxic nAg treatments used in this work caused mitochondrial oxidative stress or effected mitochondrial function, the level of mitochondrial oxidative stress and the effects on mitochondrial function by nAg in the HIEC-6 and HCT116 cells was measured.

3.7.1 Mitochondrial Oxidative Stress

In the HIEC-6 cells, mitochondrial oxidative stress slightly but non-significantly increased with 30 µg/mL PVP-nAg 4-hour treatment, and was similar to the hydrogen peroxide control. The AgNO₃ control treatments showed a slight but non-significant dose-dependent increase with 4-hour treatment (see Figure 21A). Slight but non-significant increases in mitochondrial oxidative stress were also seen when the cells were treated with nAg and AgNO₃ for 24 hours in both normoxic and hypoxic conditions (see Figure 21B and Figure 21C).

Mitochondrial oxidative stress was significantly induced in the HCT116 cells with 24-hour nAg and AgNO₃ treatment in normoxic conditions at the higher treatment levels of 20 and 30 µg/mL nAg and 2 µg/mL AgNO₃ (see Figure 22B). No significant change in oxidative stress was observed with the nAg treatments at 4 hours (see Figure 22A). Interestingly, 24-hour hypoxic treatment removed the significant increase in oxidative stress induced by nAg that was observed with the 24-hour normoxic treatments. However, a significant increase was still observed with the 2 µg/mL AgNO₃ treatment (see Figure 22C).
A

B
Figure 21: Measurement of mitochondrial oxidative stress in HIEC-6 cells using MitoSOX after 4 or 24 hours of treatment with nAg or AgNO₃ in normoxic or hypoxic conditions. A) 4-hour treatment in normoxic conditions, B) 24-hour treatment in normoxic conditions, and C) 24-hour treatment in hypoxic conditions. The values are expressed relative to the untreated control and are the mean of 3 to 6 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
C

**Figure 22:** Measurement of mitochondrial oxidative stress in HCT116 cells using MitoSOX after 4 or 24 hours of treatment with nAg or AgNO₃ in normoxic or hypoxic conditions. A) 4-hour treatment in normoxic conditions, B) 24-hour treatment in normoxic conditions, and C) 24-hour treatment in hypoxic conditions. The values are expressed relative to the untreated control and are the mean of 3 to 6 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.7.2 Seahorse Experiments to Measure Mitochondrial Response

3.7.2.1 Cell Mitochondrial Stress Test

Due to the increase in mitochondrial oxidative stress found in the HCT116 cells with nAg treatment, Seahorse experiments were conducted to determine whether impaired mitochondrial function also occurred.

Nanosilver or AgNO₃ 4-hour treatment caused no significant change in basal respiration, maximal respiration, ATP production, spare respiratory capacity, non-mitochondrial oxygen consumption, proton leak, or coupling efficiency in either the HIEC-6 (see Figure 23) or HCT116 cells (see Figure 24). Thus, no significant effect on oxidative phosphorylation was observed.

It was seen that untreated non-cancer HIEC-6 cells have a much higher resilience and spare respiratory capacity of 302.0 ± 14.9%, in comparison to untreated HCT116 colon cancer cells which have a spare respiratory capacity of 132.7 ± 3.5% (see Figure 23C and Figure 24C).
Figure 23: Oxygen consumption rate (OCR) in HIEC cells treated for 4 hours with PVP-nAg and AgNO₃ measured with the Seahorse Cell Mito Stress Test. (A) Function of oxidative phosphorylation through successive injections of oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and a combination of Rotenone and Antimycin A. (B) Oxygen consumption rate for basal respiration, maximal respiration, ATP production, spare respiratory capacity, non-mitochondrial oxygen consumption, and proton leak. (C) The spare respiratory capacity expressed as a percentage. (D) The coupling efficiency expressed as a percentage. The values are the mean of 3 three independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 24: Oxygen consumption rate (OCR) in HCT116 cells treated for 4 hours with PVP-nAg and AgNO₃ measured with the Seahorse Cell Mito Stress Test. (A) Function of oxidative phosphorylation through successive injections of Oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and a combination of Rotenone and Antimycin A. (B) Oxygen consumption rate for basal respiration, maximal respiration, ATP production, spare respiratory capacity, non-mitochondrial oxygen consumption, and proton leak. (C) The spare respiratory capacity expressed as a percentage. (D) The coupling efficiency expressed as a percentage. The values are the mean of three independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.7.2.2 Cell Energy Phenotype Tests

The cell energy phenotype test was performed to determine the effect of nAg and AgNO₃ on mitochondrial function over time. Treatment of HIEC-6 cells with 20 µg/mL PVP-nAg or 1.78 µg/mL AgNO₃ caused no significant change in OCR over the time tested (see Figure 25). In HCT116 cells, treatment with 10 µg/mL nAg and 1.3 µg/mL caused no significant change in OCR over the treatment time (see Figure 26A). Treatment of HCT116 cells with 20 µg/mL nAg also showed no significant change, however, treatment with 1.78 µg/mL AgNO₃ caused a decrease in OCR (see Figure 26B). Treatment of HCT116 cells with 50 µg/mL nAg and 2.2 µg/mL AgNO₃ resulted in an obvious decrease in OCR and was toxic to the cells. Interestingly, this nAg treatment concentration remained below the 24-hour EC₅₀ level for the cells of 78.43 ± 0.70 µg/mL. Since the AgNO₃ controls had a greater effect than their corresponding nAg concentrations (see Figure 26), it appears that the estimated AgNO₃ control concentrations of 1.78 µg/mL for 20 µg/mL nAg and 2.2 µg/mL for 50 µg/mL nAg may be an overestimation of the level of silver ions that are actually released from the nAg at these concentrations.

Figure 25: Cell energy phenotype test measuring the oxygen consumption rate (OCR) in HIEC-6 cells at baseline levels and then following injection of 20 µg/mL PVP-nAg or 1.78 µg/mL AgNO₃ at 30 minutes. The values are from one experiment in duplicate ± S.E.M.
Figure 26: Cell energy phenotype test measuring the oxygen consumption rate (OCR) in HCT116 cells at baseline levels and then following injection of PVP-nAg or AgNO$_3$ at 30 minutes with varying concentrations of PVP-nAg and AgNO$_3$. (A) 10 µg/mL nAg and 1.3 µg/mL AgNO$_3$, (B) 20 µg/mL nAg and 1.78 µg/mL AgNO$_3$, (C) 50 µg/mL nAg and 2.2 µg/mL AgNO$_3$. The values are from one experiment in duplicate ± S.E.M.
3.8 Effects of Nanosilver and Sodium Ascorbate on Cellular Stress Response Pathways

3.8.1 Cell Viability with TBHQ, SFN, and TCDD Treatment

To find non-toxic control treatment concentrations for the ARE and DRE cellular response pathways, the cell viability for TBHQ, SFN, and TCDD was examined using the MTT assay.

The cell viability of HCT116 and HEK293T cells treated with TBHQ is shown in Figure 27. Twenty-four-hour treatment with TBHQ resulted in an EC$_{50}$ value of 53.30 ± 2.74 µM for HCT116 cells, and 53.11 ± 1.27 µM for HEK293T cells (see Figure 27 and Table 5). Six-hour treatment of HEK293T cells had a higher EC$_{50}$ value of 90.48 ± 0.93 µM TBHQ. Cell viability was seen to significantly decrease with 24-hour treatment for 50 µM TBHQ treatment in the HCT116 cells and for 40 µM TBHQ treatment in the HEK293T cells. Cell viability was found to non-significantly decrease around 30 µM TBHQ treatment (see Figure 27), thus 20 or 30 µM TBHQ treatment was used as the positive control concentration.
Figure 27: The cell viability of: A) HCT116 cells treated with TBHQ for 24 hours, and B) HEK293T cells treated with TBHQ for 6 and 24 hours as measured by the MTT cell viability assay. The data is the mean of 3 to 9 individual experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
The cell viability of HCT116 cells treated with SFN is shown in Figure 28. Twenty-four-hour treatment with SFN resulted in an EC\textsubscript{50} value of 21.71 ± 0.81 µM (see Figure 28 and Table 5). Cell viability was seen to significantly decrease with 10 µM SFN, whereas 5 µM SFN treatment did not significantly decrease the cell viability. Thus, 5 µM SFN was used for the positive control treatment concentration.

![Graph showing cell viability of HCT116 cells treated with SFN](image)

**Figure 28:** Cell viability of HCT116 cells treated with SFN for 24 hours as measured by the MTT cell viability assay. The data is the mean of 4 individual experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
The cell viability of HCT116 and HEK293T cells treated with TCDD is shown in Figure 29. Twenty-four-hour treatment with TCDD resulted in an EC$_{50}$ value of 127.04 ± 3.15 nM TCDD for HCT116 cells, and was greater than 25 nM TCDD for HEK293T cells (see Figure 29 and Table 5). Six-hour treatment of HEK293T cells indicated that the EC$_{50}$ value was greater than 100 nM (see Figure 29B). A treatment concentration of 7.5 nM TCDD was chosen for the positive control concentration.
Figure 29: The cell viability of A) HCT116 cells treated with TCDD for 24 hours and B) HEK293T cells treated with TCDD for 6 and 24 hours as measured by the MTT cell viability assay. The data is the mean of 3 to 5 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
Table 5: EC₅₀ values for HCT116 and HEK293T cells treated with TBHQ, SFN, and TCDD.

<table>
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<th>EC₅₀ Values</th>
<th>HCT116</th>
<th>HEK293T</th>
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<tr>
<td>TBHQ (µM)</td>
<td>24-hour EC₅₀</td>
<td>53.30 ± 2.74</td>
<td>53.11 ± 1.27</td>
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<td>TBHQ (µM)</td>
<td>6-hour EC₅₀</td>
<td>90.48 ± 0.93</td>
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<tr>
<td>SFN (µM)</td>
<td>24-hour EC₅₀</td>
<td>21.71 ± 0.81</td>
<td></td>
</tr>
<tr>
<td>TCDD (nM)</td>
<td>24-hour EC₅₀</td>
<td>127.04 ± 3.15</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>TCDD (nM)</td>
<td>6-hour EC₅₀</td>
<td>&gt; 100</td>
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</table>

3.8.2 Effects on Protein and Gene Expression in Stress Response Pathways

3.8.2.1 Effects on Nrf1 Protein and Gene Expression

To determine whether non-toxic nAg treatment activates the ARE cellular stress response through Nrf1, the protein and gene expression of Nrf1 was examined. The Nrf1 protein expression of HCT116 cells treated for 4 hours is shown in Figure 30. No significant change in total Nrf1 protein expression was found with any of the PVP-nAg, AgNO₃, TBHQ, SFN, TCDD, hypoxic, and sodium ascorbate treatments.
Figure 30: Western blot analysis of Nrf1 protein expression profiles in response to 4 hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, TCDD, 4 hour hypoxic treatment, 4 hour hypoxic treatment combined with 20 µg/mL nAg, sodium ascorbate (SA), and SA combined with 10 µg/mL nAg. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
The peak Nrf1 gene expression in response to nAg treatment was found to occur at 4 hours (see Figure 56 in Appendix A.2). A non-significant decrease in Nrf1 gene expression is observed in response to all of the treatments (see Figure 31).

Figure 31: Relative Nrf1 gene expression in HCT116 cells treated for 4 hours as analyzed by qRT-PCR. The values were normalized to GAPDH expression. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.8.2.2 Effects on Nrf2 Protein and Gene Expression

The protein and gene expression of Nrf2 was examined in order to determine whether non-toxic nAg treatment activates the ARE cellular stress response through Nrf2.

The peak Nrf2 protein expression in HCT116 cells in response to 20 µM TBHQ treatment was found to occur after 4 hours of treatment (see Figure 55 in Appendix A.1). Four hours of nAg treatment significantly induced nuclear Nrf2 protein expression in a dose-dependent manner, with the increase in expression being significant for 10 and 20 µg/mL nAg (see Figure 32).

Silver nitrate treatment induced an increasing trend in nuclear Nrf2 protein expression, but this increase was not significant. The TBHQ and SFN positive control treatments both significantly induced nuclear Nrf2 protein expression, while TCDD did not significantly increase Nrf2 protein expression (see Figure 32).

Good separation of the cellular and nuclear fractions is demonstrated by the laminB protein expression observed for the nuclear samples but not for the cytosolic samples, and β-tubulin protein expression observed for the cytosolic samples but not for the nuclear samples.
Figure 32: Western blot analysis of Nrf2 nuclear and cytosolic protein expression profiles in response to 4-hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, and TCDD. The LaminB protein expression for the nuclear samples and the β-tubulin protein expression for the cytosolic samples are shown. The values are expressed relative to the untreated control and are the mean of 5 to 7 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
The peak Nrf2 gene expression in response to nAg treatment was found to occur at 4 hours (see Figure 56 in Appendix A.2). No significant changes in Nrf2 gene expression were found for all the treatments tested (see Figure 33).

**Figure 33:** Relative Nrf2 gene expression in HCT116 cells treated for 4 hours as analyzed by qRT-PCR. The values are normalized to GAPDH expression. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.8.2.3 Luciferase Reporter ARE Activation

Antioxidant response element transcriptional activity was examined using an ARE luciferase reporter plasmid with overexpression of Nrf1 and Nrf2.

Overexpression of Nrf2 showed that there was an increasing trend in ARE activation with the nAg treatment through Nrf2, and significant ARE activation was seen with the positive TBHQ control treatment (see Figure 34). Overexpression of Nrf1 with the N-terminal FLAG tagged Nrf1 plasmid had a significant decrease in activation with the nAg and AgNO₃ treatments while still showing a significant increase in activation with the positive TBHQ control (see Figure 35). No significant change in activation was seen with the nAg and AgNO₃ treatments with overexpression of the C-terminal FLAG tagged Nrf1 plasmid, however, the positive TBHQ control showed a significant increase in activation (see Figure 34). Thus, the luciferase reporter assays indicated that nAg treatment induces activation of the ARE detoxification pathway through Nrf2 as opposed to Nrf1.
Figure 34: Luciferase reporter assay analysis of Nrf2 transactivation activity of the pGL4.37 ARE luciferase reporter in HEK293T cells with pCR3.1 as the empty plasmid control in response to 24-hour treatment with PVP-nAg, AgNO₃, and TBHQ for the control. The values are expressed relative to the untreated control and are the mean of 5 to 8 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
**Figure 35:** Luciferase reporter assay analysis of N-Nrf1 and CMV-Nrf1 transactivation activity of the pGL4.37 ARE luciferase reporter in HEK293T cells in response to 24-hour treatment with PVP-nAg, AgNO₃, and TBHQ for the control. The values are expressed relative to the untreated control and are the mean of 3 to 4 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.8.2.4 Effects on AhR Protein and Gene Expression

The protein and gene expression of AhR was examined to ascertain whether non-toxic nAg treatment activates the DRE cellular stress response through AhR.

The nuclear and cytosolic AhR protein expression of HCT116 cells treated for 4 hours is shown in Figure 36. No significant change was found in the nuclear AhR protein expression with the treatments, however, an increasing trend was seen. The cytosolic AhR protein expression was significantly increased with 20 µg/mL PVP-nAg treatment (see Figure 36). Good separation of the cellular and nuclear fractions was demonstrated by the LaminB protein expression observed for the nuclear samples but not for the cytosolic samples, and β-tubulin protein expression observed for the cytosolic samples but not for the nuclear samples.
Figure 36: Western blot analysis of AhR nuclear and cytosolic protein expression profiles in response to 4-hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, and TCDD. The LaminB protein expression for the nuclear samples and the β-tubulin protein expression for the cytosolic samples are shown. The values are expressed relative to the untreated control and are the mean of 2 – 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
The peak AhR gene expression in response to nAg treatment was found to occur at 4 hours (see Figure 56 in Appendix A.2). No significant change was observed in the AhR gene expression in HCT116 cells with 4-hour treatment (see Figure 37).

![Figure 37:](image)

**Figure 37:** Relative AhR gene expression in HCT116 cells treated for 4 hours as analyzed by qRT-PCR. The values were normalized to GAPDH expression. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.8.2.5 Luciferase Reporter DRE Activation

Dioxin response element transcriptional activity was examined in this study using a DRE luciferase reporter plasmid with AhR overexpression. No significant change was found, although a slight non-significant decrease was observed with the PVP-nAg and AgNO$_3$ treatments. The TCDD positive control significantly increased DRE activity (see Figure 38).

**Figure 38:** Luciferase reporter assay analysis of AhR transactivation activity of the p1A1 DRE luciferase reporter with pCR3.1 as the empty plasmid control in response to 24-hour treatment with PVP-nAg, AgNO$_3$, TCDD, and TBHQ. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.8.2.6  **Effects on HIF-1α Protein Expression**

To discover the effects of nAg, sodium ascorbate, and combined treatments of nAg and sodium ascorbate on the hypoxic stress response, the protein expression of HIF-1α was examined.

Four-hour 20 µg/mL PVP-nAg and 1.32 µg/mL AgNO₃ treatment significantly induced HIF-1α protein expression under normoxic conditions (see Figure 39). Hypoxic treatment strongly induced HIF-1α protein expression as a positive control. Sulforaphane also significantly induced HIF-1α protein expression. Treatment with sodium ascorbate both on its own and when combined with nAg decreased HIF-1α protein expression, although this was not significant.
**Figure 39:** Western blot analysis of HIF-1α protein expression profile in response to 4-hour treatment with PVP-nAg, AgNO$_3$, TBHQ, SFN, TCDD, 4-hour hypoxic treatment, 4-hour hypoxic treatment combined with 20 µg/mL nAg, sodium ascorbate (SA), and SA combined with 10 µg/mL nAg. The values are expressed relative to the untreated control and are the mean of 3 to 4 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.9 Nanosilver and ER Stress Response Pathways

Depending on dose, treatment time, size of nAg, and the sensitivity of the cells, ER stress may be induced in the cells by nAg treatment. Thus, the effects of non-toxic nAg treatment on the ER stress UPR pathways involving XBP1 and ATF4 were examined.

3.9.1 Effects on ER Stress Through XBP1

The effect of PVP-nAg treatment on ER stress through XBP1 was examined using flow cytometry and HCT116 cells stably transfected with XBP1. Positive control treatment with 200 nM Thapsigargin for 24 hours was found to give the greatest induction in fluorescent intensity (see Figure 40).
Figure 40: Treatment time course of the stably transfected HCT116-XBP1 cells treated in normoxic conditions. The values are from 1 to 5 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.

There was no significant induction of ER stress through XBP1 seen with the PVP-nAg treatment at 16-hour (see Figure 41) or 24-hour (see Figure 42) treatment in normoxic conditions. The higher AgNO₃ concentrations at 1.5 and 2 μg/mL significantly induced ER stress through XBP1 at 24 hours, with similar results occurring in both normoxic and hypoxic conditions (see Figure 42).
Figure 41: Relative Fluorescent Intensity from 16-hour treatment of stably transfected HCT116-XBP1 cells in normoxic conditions. The values are from 3 to 5 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test: * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 42: Relative Fluorescent Intensity from 24-hour treatment of stably transfected HCT116-XBP1 cells in normoxic and hypoxic conditions. The values are from 4 to 8 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.9.2 Effects on ER Stress Through ATF4

The effects of nAg treatment on ER stress through ATF4 were examined using flow cytometry and HCT116 cells stably transfected with ATF4. Positive control treatment with 200 nM Thapsigargin for 24 hours was found to give the greatest induction in fluorescent intensity (see Figure 43).

![Figure 43](image)

**Figure 43**: Treatment time course of the stably transfected HCT116-ATF4 cells treated in normoxic conditions. The values are from 1 to 9 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
There was no significant induction of ER stress through ATF4 with the 24-hour nAg treatment. The highest tested AgNO₃ concentrations of 2 µg/mL AgNO₃ significantly induced ER stress through ATF4 at 24 hours in normoxic conditions (see Figure 45). Weaker response and no significant induction was seen with 16 hours of treatment (see Figure 44).

**Figure 44:** Relative Fluorescent Intensity from 16-hour treatment of stably transfected HCT116-ATF4 cells in normoxic conditions. The values are from 3 to 8 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
**Figure 45:** Relative Fluorescent Intensity from 24-hour treatment of stably transfected HCT116-ATF4 cells in normoxic and hypoxic conditions. The values are from 4 to 9 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.10 Effects of Nanosilver and Sodium Ascorbate on WNT Signalling Pathways

The effects of nAg, sodium ascorbate, and combined treatments on WNT signalling were examined. This is important since over-activation of WNT signaling has been found in colon cancer but is not well understood.

3.10.1 Effects on PORCN Protein and Gene Expression

Porcupine is required for the palmitoleoylation of WNT proteins in WNT signalling. The PORCN protein expression of HCT116 cells treated for 4 hours is shown in Figure 46. No significant change was found in PORCN expression with the treatments of PVP-nAg, AgNO₃, or nAg combined with sodium ascorbate (see Figure 46). Sulforaphane treatment significantly increased PORCN protein expression (see Figure 46).
Figure 46: Western blot analysis of PORCN protein expression profiles in response to 4-hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, TCDD, hypoxic treatment, hypoxic treatment combined with 20 µg/mL nAg, sodium ascorbate (SA), and SA combined with 10 µg/mL nAg. The values are expressed relative to the untreated control and are the mean of 4 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.

The peak PORCN gene expression in response to nAg treatment was found to occur around 4 – 6 hours (see Figure 56 in Appendix A.2). No significant changes in PORCN gene expression was observed for all the treatments at 4 hours (see Figure 47).
Figure 47: Relative PORCN gene expression in HCT116 cells treated for 4 hours as analyzed by qRT-PCR. The values were normalized to GAPDH expression. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.

3.10.2 Effects on WNTless Protein and Gene Expression

After palmitoleoylation, the membrane transport protein WNTless carries WNT from the ER to the plasma membrane for secretion. The WNTless protein expression of HCT116 cells treated for 4 hours is shown in Figure 48. No significant increases were found, however, a dose-dependent increase in WNTless is seen with increasing nAg treatment concentrations. No change
in WNTless expression was seen with sodium ascorbate or combined nAg and sodium ascorbate treatment (see Figure 48).

**Figure 48:** Western blot analysis of WNTless protein expression profiles in response to 4-hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, TCDD, hypoxic treatment, hypoxic treatment combined with 20 µg/mL nAg, sodium ascorbate (SA), and SA combined with 10 µg/mL nAg. The values are expressed relative to the untreated control and are the mean of 3 to 4 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
The peak WNTless gene expression in response to nAg treatment was found to occur at 4 hours (see Figure 56 in Appendix A.2). No significant changes in WNTless gene expression was observed for all the treatments at 4 hours (see Figure 49).

**Figure 49:** Relative WNTless gene expression in HCT116 cells treated for 4 hours as analyzed by qRT-PCR. The values were normalized to GAPDH expression. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.10.3 Effects on WNT5A Protein and Gene Expression

Since WNT5A is part of the WNT signalling β-catenin-independent pathway, the protein and gene expression of WNT5A was examined.

The WNT5A protein expression of HCT116 cells treated for 4 hours is shown in Figure 50. No significant change was found in the WNT5A protein expression with the nAg and AgNO₃ treatments. Hypoxic treatment, sodium ascorbate treatment, and co-treatment of nAg with sodium ascorbate all displayed a decreasing, but non-significant, trend in WNT5A protein expression to a level lower than the untreated control (see Figure 50).

The WNT5A gene expression of HCT116 cells treated for 4 hours is shown in Figure 51. No significant changes in WNT5A gene expression were observed for all the treatments tested.
Figure 50: Western blot analysis of WNT5A protein expression profiles in response to 4-hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, TCDD, hypoxic treatment, hypoxic treatment combined with 20 µg/mL nAg, sodium ascorbate (SA), and SA combined with 10 µg/mL nAg. The values are expressed relative to the untreated control and are the mean of 2 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
**Figure 51:** Relative WNT5A gene expression in HCT116 cells treated for 4 hours as analyzed by qRT-PCR. The values were normalized to GAPDH expression. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.10.4 Effects on WNT3A Protein and Gene Expression

The protein and gene expression of WNT3A was examined in order to determine the effects on the WNT signalling β-catenin-dependent pathway. The WNT3A protein expression of HCT116 cells treated for 4 hours is shown in Figure 52. No significant changes were seen in WNT3A protein expression across all the treatments.

![Western blot analysis of WNT3A protein expression profiles](image)

**Figure 52:** Western blot analysis of WNT3A protein expression profiles in response to 4-hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, TCDD, hypoxic treatment, hypoxic treatment combined with 20 µg/mL nAg, sodium ascorbate (SA), and SA combined with 10 µg/mL nAg. The values are expressed relative to the untreated control and are the mean of 2 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
The WNT3A gene expression of HCT116 cells treated for 4 hours is shown in Figure 53.

No significant changes in WNT3A gene expression were observed for all the treatments tested.

![Relative WNT3A Gene Expression](image)

**Figure 53:** Relative WNT3A gene expression in HCT116 cells treated for 4 hours as analyzed by qRT-PCR. The values were normalized to GAPDH expression. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.

### 3.10.5 Effects on β-catenin Protein Expression

In the β-catenin-dependent pathway, β-catenin accumulates and translocates to the nucleus. The effects on the β-catenin-dependent pathway was examined by measuring the protein and gene expression of β-catenin. The β-catenin protein expression of HCT116 cells treated for 4
hours is shown in Figure 54. No significant effect was observed in β-catenin protein expression with all treatments (see Figure 54).

**Figure 54:** Western blot analysis of β-catenin protein expression profiles in response to 4-hour treatment with PVP-nAg, AgNO₃, TBHQ, SFN, TCDD, hypoxic treatment, hypoxic treatment combined with 20 µg/mL nAg, sodium ascorbate (SA), and SA combined with 10 µg/mL nAg. The values are expressed relative to the untreated control and are the mean of 3 to 4 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
Chapter 4: Discussion

Nanosilver has superb antimicrobial, antiviral, antifungal, antiparasitic, and anticancer properties and is being increasingly used in many commercial, medical, and household applications. Thus, human exposure to nAg is steadily increasing, with much remaining to be discovered about the interactions of nAg in the human body and its beneficial or toxic effects both alone or in combination with other compounds. Depending on work, lifestyle, and medical treatments, human exposure to nAg can occur through various avenues including inhalation, ingestion, skin contact, or other means. The exposure route determines which organs and tissues are most exposed. It is important to note that the physical characteristics of the nAg including size, shape, surface coating, surface charge, protein corona formation, and aggregation state all affect the exposure level and biological responses to the nAg.

Nanosilver treatment of cancer has had promising results in both cell culture and animal studies, as well as in a case study where nAg treatment resulted in spontaneous remission in the patient. Nanosilver has also shown promising results when combined with radiation treatment.

Sodium ascorbate is a vital water-soluble antioxidant that can neutralize the free radicals and ROS that are produced during sickness. It also has pro-oxidant effects in its oxidized form, and in this form is able to kill cancer cells through the production of hydrogen peroxide. There is mounting evidence for the benefit and safety of intravenous sodium ascorbate in increasing the effectiveness of radiation and chemotherapy treatments, while at the same time decreasing, or at the very least not adding to, the detrimental side effects. Both nAg and ascorbate have shown promising anti-cancer effects although much remains to be discovered.

With the growing prevalence of cancer in people, there is increased need to find both new treatments and combinations of treatments to further treatment effectiveness. Colon cancer is the
second most common cancer worldwide and advanced colon cancer is difficult to treat successfully with conventional treatments due to developed drug resistance and the occurrence of metastasis (Chakrabarti et al., 2020; Wen et al., 2019). Since oral ingestion brings nAg directly into the gastrointestinal tract and sodium ascorbate can be administered through intravenous treatment, nAg and sodium ascorbate can be brought directly to or near the site of concern.

4.1 Nanosilver Characterization

Characterization of the nAg that is used in the treatments is important in understanding what the cells are being treated with and in comparing the observed effects to results in the literature. The metallic-core size, nanoparticle shape, surface stability, capping agent, and silver concentration of the colloidal solution are all important parameters to consider in this respect (Duval et al., 2019). Care must be taken with the handling methods since different handling and dilution methods may also affect the aggregation state of the nAg (Huang et al., 2015).

In this work, the metallic-core size of the 10 nm PVP-nAg obtained from NanoComposix was measured by TEM and was found to be very close to the desired size at 9.99 ± 0.37 nm (see Figure 5). The PVP-nAg particles were suspended in purified water and were seen to be single spheres by TEM (see Figure 6). The measured UV-Vis spectrum showed one peak for un-aggregated nAg, with a secondary peak being observed when aggregation occurred (see Figure 7), however, only unaggregated nAg was used in this work. The hydrodynamic diameter of the PVP-nAg was 19 nm (reported by NanoComposix for product number: AGPO10, lot number: SAM0384). Nanosilver surface stability was maintained with the use of 40 kDa PVP polymer as the capping agent. The measured zeta potential was -25 mM, the actual zeta potential may be greater due to inaccuracies caused by low concentration (reported by NanoComposix for product
number: AGPO10, lot number: SAM0384). Particles in a colloidal solution with large positive or negative zeta potentials greater than 30 mV will repel each other, remain unaggregated, and are considered stable (Duval et al., 2019). The silver concentration of the PVP-nAg solution used was 1.01 mg/mL, $1.4 \times 10^{14}$ particles/mL, and $2.3 \times 10^{-7}$ mol/L (reported by NanoComposix for product number: AGPO10, lot number: SAM0384).

4.2 Determining the AgNO$_3$ Control Treatment Concentrations

Nanosilver releases silver ions when its surface is oxidized. It is important to control for the silver ions released in the treatments in order to distinguish between nAg-specific effects and effects that are a result of the released silver ions. The release of silver ions from nAg has been referred to as a “Trojan horse” effect, where the toxicity is thought to be mainly through the released silver ions and resulting oxidative stress (Buttacavoli et al., 2018; Chen et al., 2014a; Cronholm et al., 2013; Wang et al., 2013). However, the nanosilver particles themselves also have nanoparticle specific effects independent of their released silver ions. The size of the nAg particles, the pH of the environment they are in, and the presence of various surface coatings or a protein cornea all affect the rate of silver ion release (Durán et al., 2015; Manshian et al., 2015; Mao et al., 2016b; Qian et al., 2015). Smaller nAg particles have been found to have a higher rate of silver ion release than larger nanoparticles (Karlsson et al., 2014). This phenomenon may be due to the increased curvature of the smaller nAg particles. Silver nitrate is the most commonly used silver ion control in the literature, with silver acetate or silver carbonate occasionally being used (Greulich et al., 2011; Kawata et al., 2009; Nallanthighal et al., 2017; Prasad et al., 2013a). In this study, AgNO$_3$ was used as the silver ion positive control.
In order to measure the concentration of silver ions released from nAg in a certain condition, it is a common technique to remove the silver nanoparticles from the solution by centrifugation and then measure the concentration of silver ions left behind in the supernatant by ICP-MS (Lin et al., 2014; Manshian et al., 2015; Wang et al., 2013; Zielinska et al., 2018). Silver has an atomic weight of 107.8, with two isotopes of 107 (52%) and 109 (48%) (Panyala et al., 2008).

The concentration of silver ions released from PVP-nAg and AgNO₃ in media as measured by ICP-MS, and the calculated equivalent AgNO₃ concentrations are shown in Figure 7 and Table 3. This experiment was done with a replicate of one, which is not as accurate as it would be with more replicates. Future work would include performing more replicates of this experiment, as well as measuring the silver ions released from higher nAg concentrations so the equivalent AgNO₃ concentrations could be measured rather than estimated.

The AgNO₃ control concentrations for 24-hour treatment were estimated from the EC₅₀ value of the cells (see Table 4) to allow for cell viability in the experiments. The estimated equivalent AgNO₃ concentrations for 24-hour nAg treatment from the equations shown in Figure 7 were unreasonably high (data not shown) and would have been unduly toxic to the cells. Such toxicity was deemed unrealistic since the nAg concentrations they were intended to be a control for were not themselves toxic to the cells. This adjustment in AgNO₃ control treatment concentrations at 24 hours was seen to be more in line with the actual level of silver ions being released from the nAg particles during the experiments.

The AgNO₃ control concentration for the Cell Mito Stress Test Seahorse experiment was chosen to be 1.65 µg/mL AgNO₃ to correspond to 20 µg/mL PVP-nAg (see Figure 23 and Figure
24). This was used instead of 1.78 $\mu$g/mL AgNO$_3$, since this was found to be more toxic to the HCT116 cells than 20 $\mu$g/mL PVP-nAg in the cell energy phenotype test (see Figure 26).

4.3 **Cell Viability with nAg, AgNO$_3$, and SA Treatments**

The cell viability of various cell lines was assessed in this study using the MTT assay. This assay relies on the activity of the mitochondrial enzymes succinate dehydrogenase and NADH- and NADPH-quinone oxidoreductase to reduce the MTT to a purple formazan salt that is water insoluble but can be dissolved in DMSO and the relative amount determined by the absorbance at 570 nm. This assay assumes that the mitochondrial activity is constant in the cells and thus corresponds to the number of viable cells (Collier & Pritsos, 2003).

A hormetic effect can be observed occurring in the cells where low dose treatment stimulates a higher cellular response than the untreated control, as is seen in Figure 9, Figure 11 and Figure 13. This is due to the fact that low exposure levels trigger cell-survival pathways, and this has been found in other studies to be protective for the cells against subsequent higher dose treatment (Jiao et al., 2014; Mao et al., 2016a; Sthijns et al., 2017).

4.3.1 **Cell Viability with nAg Treatments**

Out of all the cell lines tested, the viability of the HIEC-6 non-cancer colon cells was not decreased by the PVP-nAg. In comparison, the viability of the HCT116 colon cancer cells significantly decreased with the PVP-nAg treatment (see Figure 9, Figure 10, and Table 4). In fact, the PVP-nAg was found to be non-toxic to the HIEC-6 cells at the treatment concentrations tested up to 125 $\mu$g/mL for 24 hours. In comparison to these cells, cytotoxicity was found with the PVP-nAg treatment of the HCT116 colon cancer cells (EC$_{50}$ value of 78.43 ± 0.70 $\mu$g/mL
when treated for 24 hours). The HIEC-6 cells were the least affected by the AgNO₃ (EC₅₀ value of 17.97 ± 0.36 µg/mL when treated for 24 hours), while the HCT116 cells showed a much higher toxicity (1.64 ± 0.03 µg/mL when treated for 24 hours).

In other cell line studies, nAg has consistently been found to be more toxic to cancer cells than to non-cancer cells, demonstrating decreased cell viability, cell cycle arrest, increased DNA damage, decreased mitochondrial function, increased ROS, and increased cell membrane permeability. This has been observed with MCF-7 and T-47D breast cancer cells treated with 2 and 15 nm nAg compared to MCF-10A non-cancer breast cells (Simard et al., 2016); in MCF-7, MDA-MB 231 breast cancer cells, A549 lung carcinoma cells, and 2780 human ovarian cancer cells treated with 10 nm nAg compared to L132 non-cancerous lung epithelial cells (Jeong et al., 2016); in human liver carcinoma HepG2 cells treated with 23.44 nm nAg compared to non-cancerous hepatic L02 cells (Xue et al., 2018); and in cancerous U251 human glioblastoma cells treated with 6 – 20 nm nAg compared to non-cancerous IMR-90 cells (Asharani et al., 2009).

The HEK293T cells had the largest decrease in cell viability with the PVP-nAg treatment, with longer treatment times resulting in more cell death than shorter treatment times. Since 6-hour treatment is less toxic to the cells than 24-hour treatment and since there were no changes in the 24-hour treatment times at the lower concentrations, therefore some of the 6-hour treatments at lower concentrations were not tested.

Citrate-nAg was found to be more toxic than PVP-nAg to the HEK293T cells (see Figure 9 and Table 4). This may be due to higher silver ion release or due to increased nAg internalization, as was observed in HepG2 cells (Prasad et al., 2013a).
4.3.2 Cell Viability with AgNO$_3$ Treatments

Similar to the PVP-nAg results, the AgNO$_3$ treatment was least toxic to the HIEC-6 non-cancer colon cells, while the viability of the HCT116 colon cancer cells significantly decreased when compared to the viability of the HIEC-6 cells with AgNO$_3$ treatment (see Figure 11, Figure 12, and Table 4).

The HEK293T cells had the largest decrease in cell viability with the AgNO$_3$ treatment, with longer treatment times resulting in more cell death than shorter treatment times. Since 6-hour treatment is less toxic to the cells than 24-hour treatment and since there were no changes in the 24-hour treatment times at the lower concentrations, therefore some of the 6-hour treatments at lower concentrations were not tested.

The cell viability of the SH-SY5Y and RAW264.7 cells was slightly more than that of the HEK293T and HCT116 cells, but was still below that of the HIEC-6 cells (see Figure 11 and Table 4).

4.3.3 Cell Viability with SA Treatments, and nAg and SA Combined Treatments

Sodium ascorbate was used to treat the cells rather than ascorbic acid as this is the buffered form of vitamin C. The viability of the HIEC-6 cells was significantly lower compared to the HCT116 cells when treated with sodium ascorbate for 24 hours (see Figure 13, Figure 14, and Table 4). This result was not expected and may be due to the specific cell lines used. In a study by Chen et al. (2005), normal cells tested (lymphocytes and monocytes from healthy volunteers, breast Hs587Bst cells, and fibroblast CCD34SK cells) had no decreased cell viability even at 20 mM ascorbate when they were treated for 1 hour, washed, and allowed to recover for 18 – 22 hours (possibly simulating the relatively quick clearance of vitamin C from the body);
while all nine cancer cell lines tested had lower EC\textsubscript{50} values, with five of these having EC\textsubscript{50} values below 5 mM (Chen et al., 2005; Lykkesfeldt & Tveden-Nyborg, 2019).

Low-dose treatment levels of 10 µg/mL PVP-nAg and 1 mM sodium ascorbate were chosen for the combined treatments since these levels were non-toxic to both cell lines on their own (see Figure 9A & Figure 9B, and Figure 13A & Figure 13B). Interestingly, combined treatments of sodium ascorbate with PVP-nAg showed increased lethality to the HCT116 cells as opposed to the HIEC-6 cells, and in particular, low-dose PVP-nAg combined with sodium ascorbate treatments increased the HCT116 cell death from that observed with either the nAg or the sodium ascorbate treatments alone (see Figure 15).

Treatment with constant 10 µg/mL PVP-nAg and increasing concentrations of sodium ascorbate resulted in a greatly decreased EC\textsubscript{50} value for the HCT116 cells from 5.37 ± 0.92 mM sodium ascorbate to 0.73 ± 0.20 mM sodium ascorbate. The HIEC-6 cells experienced less of a decrease in cell viability when the nAg was combined with the varying sodium ascorbate treatments, and went from an EC\textsubscript{50} value of 3.77 ± 0.01 mM for the 24-hour sodium ascorbate treatment to 1.71 ± 0.05 mM with the combined treatment, which was higher than that for the HCT116 cells. Thus, the combined nAg and sodium ascorbate treatments resulted in higher toxicity in the HCT116 cells as compared to the HIEC-6 cells.

On the other hand, low-dose sodium ascorbate combined with PVP-nAg treatments still decreased the viability of the HCT116 cells, however, the EC\textsubscript{50} value of the HCT116 cells was higher than with just the PVP-nAg treatment on its own. Incorporating a constant low-dose 1 mM sodium ascorbate treatment with varying nAg treatments resulted in increased toxicity of the HCT116 cells while no decrease in viability was observed in the HIEC-6 cells. Interestingly, although this combined treatment still decreased the viability of the HCT116 cells, the EC\textsubscript{50}
value was not found since it was higher than 100 µg/mL nAg with the added 1 mM sodium ascorbate. This indicated that the relatively low sodium ascorbate supplementation in fact helped the HCT116 cells to survive the high nAg treatment, since with just the nAg treatment alone the EC$_{50}$ value was 78.43 ± 0.70 µg/mL nAg (see Figure 13D and Table 4).

Thus, in terms of combined treatments, it is much more advantageous to use a higher sodium ascorbate treatment level with low nAg treatment than to use low sodium ascorbate with high nAg treatment. This is a novel result that has not been reported before in the literature.

The protective effect of low dose sodium ascorbate from the toxicity of high dose nAg has been observed in vitro and in vivo. Cotreatment with ascorbate decreased the toxicity of 20 nm nAg on human embryonic stem cell (hESC) derived neural progenitor cells (Li et al., 2021). In vivo, rats treated via intraperitoneal injection with nAg every day for 28 days as well as having ascorbate in their drinking water did better than the rats treated with just the nAg. The rats given ascorbate experienced less toxicity and less histological damage than the rats only treated with nAg and not given the ascorbate. Histological and mitochondrial degeneration was observed in these latter rats, as well as having the rough ER and lysosomes visibly swollen and filled with nAg particles (Taghyan et al., 2020).

4.4 Cell Pictures

The visible health of the treated cells corresponded to the cell viability that was observed with the MTT assays. As seen in Figure 16, the HIEC-6 cells appeared visibly healthy at non-toxic treatment concentrations well below the EC$_{50}$ for nAg and AgNO$_3$. For the HCT116 cells, toxicity was observed near and above the EC$_{50}$ for nAg and Ag NO$_3$. Additionally, nanosilver
was seen to be inside both the HIEC-6 and the HCT116 cells at 30 µg/mL PVP-nAg, which was below the EC₅₀ for both of the cell lines.

4.5 Cell Cycle Analysis

The effects of nAg, AgNO₃, SA, and combined treatments of nAg and SA on the cell cycle was examined in this work in order to investigate an aspect of how cell death was being induced in the HCT116 cells. Cellular stress or DNA damage can disrupt the cell cycle resulting in an accumulation of cells in one of the following cell cycle phases: gap 1 (G1), DNA synthesis (S), or the gap 2 (G2)/mitosis (M) phase. This serves the purpose of preventing replication of damaged DNA and allows the cell time to make repairs; or if the damage is too great and the cell cannot recover leads to cell death. An accumulation of cells in the subG1 phase is indicative of apoptotic cells, potentially due to irreversible DNA damage (Oh et al., 2016).

In this work, treatment with PVP-nAg and combined SA and PVP-nAg resulted in cell cycle arrest in the G2/M phase in the HCT116 cells. This cell cycle arrest with the combined treatment was slightly more than that observed with the nAg treatment alone, although there was no significant difference between these. Sodium ascorbate on its own did not induce cell cycle arrest.

The nAg-induced cell cycle arrest was a nanoparticle-specific effect and did not occur with the AgNO₃ silver ion control treatments. The AgNO₃ did not induce cell cycle arrest even at the highest treatment concentration (see Figure 18). This indicates that the cell cycle arrest in the G2/M phase seen with the nAg treatment is a silver nanoparticle specific effect and is not the result of released silver ions or oxidative stress caused by released silver ions. Since nAg is known to have the ability to bind to proteins through their thiol groups and disrupt their structure...
and function, it is possible that the nAg, but not the silver ions, are binding to and inhibiting some aspect of one or more of the cell cycle proteins involved with the G2/M phase, resulting in the observed cell cycle arrest.

Cell cycle arrest has been reported to occur in the G2/M phase with nAg treatment. This was observed in cancerous human glioblastoma U251 cells, with these cancer cells being more highly affected than non-cancerous IMR-90 cells (Asharani et al., 2009); as well as in human renal proximal tubular epithelial HK-2 cells (Kang et al., 2012). SubG1 cell cycle arrest and increased DNA fragmentation has also been reported with nAg treatment in human embryonic stem cell-derived neural stem/progenitor cells (hESC-derived NPCs) (Oh et al., 2016).

Cell cycle progression from the G2 to the M phase requires active (phosphorylated) cyclin-dependent kinase 1 (CDK1, also called cell division cycle protein 2 homolog [CDC2]). Nanosilver treatment on human renal proximal tubular epithelial HK-2 cells has been found to decrease the levels of active CDK1. Cyclin-dependent kinase 25 (CDC25) activates CDK1; and nAg treatment was found to also decrease the levels of active (phosphorylated) CDC25 (Kang et al., 2012). The protein expression levels of p53 and p21, which are involved in cell cycle arrest due to DNA damage, both increased. These effects were increased through Nrf2 knockdown, with Nrf2 being a transcription factor involved in the cellular antioxidant response. Pre-treatment with the antioxidant NAC alleviated the observed cell cycle arrest, DNA damage, and ROS; while pretreatment with L-buthionine-[S,R]-sulfoximine (BSO), which inhibits the synthesis of GSH, exacerbated the effects (Kang et al., 2012).
4.6 Cellular Oxidative Stress

It has been thought that the biological effects of smaller nanoparticles occurs mainly through oxidative stress toxicity (Hewitt et al., 2020), however, as is seen in Figure 19 and Figure 20, no change in cellular oxidative stress was observed at non-toxic nAg treatment concentrations in either the HIEC-6 or HCT116 cells at either 4 or 24 hours in either normoxic or hypoxic conditions.

Since no change in cellular oxidative stress was observed at non-toxic nAg treatment concentrations, the cellular effects seen at this treatment level are due to factors other than the generation of oxidative stress.

4.7 Mitochondrial Oxidative Stress and Function

4.7.1 Mitochondrial Oxidative Stress

In normoxic conditions, no significant changes in mitochondrial oxidative stress were found in the HIEC-6 cells (see Figure 21); however, a significant increase in mitochondrial oxidative stress was found in HCT116 cells treated with 20 and 30 µg/mL PVP-nAg and 2 µg/mL AgNO₃ for 24-hours (see Figure 22). No change in mitochondrial oxidative stress was found with shorter 4-hour treatment (see Figure 22).

Interestingly, in 24-hour hypoxic treatment, no significant mitochondrial oxidative stress was caused by the nAg treatment but was caused by the AgNO₃ (see Figure 22). The presence of oxygen is necessary for the release of silver ions from nAg (Liu & Hurt, 2010), and thus this inhibition of mitochondrial oxidative stress may be due to a decrease in silver ions released from the nAg in the hypoxic environment. Activation of the cellular response to hypoxic stress, which was significantly induced with 4-hour 20 µg/mL PVP-nAg treatment (see Figure 39), may also
have contributed. This effect by hypoxia has been previously observed, where hypoxic treatment lessened the mitochondrial damage and lessened the decrease in mitochondrial membrane potential caused by treating A549 cells with high nAg treatment at the EC$_{50}$ level (Jeong et al., 2016).

### 4.7.2 Seahorse Experiments to Measure Mitochondrial Response

#### 4.7.2.1 Cell Mitochondrial Stress Test

The effects of nAg and AgNO$_3$ treatment on oxidative phosphorylation were examined using the cell mito stress test and the cell energy phenotype test from Agilent with the Seahorse XFp Analyzer. In the Cell Mito Stress Test, the oxygen consumption rate (OCR) was measured after 4 hours of nAg and AgNO$_3$ treatment. Successive injections of oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and rotenone with antimycin A were done. Initially, basal respiration was measured before the addition of oligomycin. Oligomycin is a complex V inhibitor, thus it removes all ATP associated respiration and causes a drop in OCR, with this drop showing the level of ATP production in the cells.

The mitochondrial maximum respiration rate was seen after the addition of FCCP, a protonophore that collapses the proton gradient across the inner mitochondrial membrane and forces the electron transport chain to its maximal rate in an attempt to restore the proton gradient. The difference in level between the basal respiration rate and the maximum respiration rate indicates the spare respiratory capacity of the cells. A combination of rotenone and antimycin A was injected last to inhibit both complex I and complex III respectively. This completely blocks oxidative phosphorylation, revealing the background level of non-mitochondrial respiration. The difference between this end level of background non-mitochondrial respiration and the level after
oligomycin was added (and ATP production inhibited) indicates the level of proton leakage back across the inner mitochondrial membrane that occurs, bypassing complex V and lowering the electron transport chain coupling efficiency (Jastroch et al., 2010).

Four-hour PVP-nAg or AgNO₃ treatment did not have any significant effect on mitochondrial function in terms of basal respiration, maximal respiration, ATP production, spare respiratory capacity, non-mitochondrial oxygen consumption, proton leak, or coupling efficiency in either the HIEC-6 (see Figure 23) or HCT116 cells (see Figure 24). It is possible that an effect on oxidative phosphorylation might occur in the cells with longer treatment or with higher treatment concentrations, where mitochondria oxidative stress was observed in the HCT116 cells (see Figure 22). Similar to the results found in this work, no significant changes have been found in basal respiration, maximal respiration, proton leak, spare respiratory capacity, or coupling efficiency in A549, BEAS-2B, Calu-1, and HCl-H358 cells treated with 10 µg/mL nAg for 1 or 24 hours (Holmila et al., 2019).

A difference between the two cell lines was observed in that the non-cancer HIEC-6 cells are more resilient and have a higher spare respiratory capacity than the HCT116 colon cancer cells (see Figure 23C and Figure 24C).

### 4.7.2.2 Cell Energy Phenotype Tests

The cell energy phenotype test was used to examine the effects of nAg and AgNO₃ treatment on OCR over time. In the cell energy phenotype test, OCR was initially measured for 30 minutes to establish a baseline level and then measured following injection of nAg and AgNO₃. No change in OCR was found over 6 hours in the HIEC-6 cells treated with 20 µg/mL PVP-nAg or 1.78 µg/mL AgNO₃ (see Figure 25). In the HCT116 cells, treatment with 20 µg/mL
PVP-nAg did not affect the OCR, however treatment with 1.78 μg/mL AgNO₃ decreased the OCR. Although more replicates are required, this may indicate a higher sensitivity to the treatment than the HIEC-6 cells (see Figure 26).

### 4.8 Effects of Nanosilver and Sodium Ascorbate on Cellular Stress Response Pathways

#### 4.8.1 Cell Viability with TBHQ, SFN, and TCDD Positive Control Treatments

In this work, TBHQ and SFN were chosen as positive controls for activation of the ARE pathway, and TCDD was chosen as the positive control for the DRE pathway. To find non-toxic control treatment concentrations, the cell viability for these chemicals was examined using the MTT assay. The cell viability and EC₅₀ values for HCT116 and HEK293T cells treated with TBHQ, SFN, and TCDD are shown in Figure 27, Figure 28, Figure 29, and Table 5.

For TBHQ, 20 or 30 μM TBHQ treatment was chosen for the positive control concentrations with this being within the treatment range used in literature (Khodagholi & Tusi, 2011; Koh et al., 2009; Lavoie et al., 2009).

In HCT116 cells, treatment with 10 μM SFN significantly decreased cell viability after 24-hours while 5 μM SFN did not (see Figure 28). Thus 5 μM SFN was chosen for the positive control treatment concentration, and was within the range of 3 – 15 μM used in literature (Agyeman et al., 2012; Kemmerer et al., 2015; Kensler et al., 2013; Kubo et al., 2017).

The cell viability EC₅₀ values for HCT116 and HEK293T cells treated with TCDD for 24-hours were 127.04 ± 3.15 nM and >25 nM TCDD, respectively (see Figure 29 and Table 5). These were well above the treatment concentration of 7.5 nM TCDD that was chosen for the positive control treatment. This was in the same range of 1 – 10 nM TCDD used in literature (He et al., 2011; Morel & Barouki, 1998).
4.8.2 Effects on Protein and Gene Expression in Stress Response Pathways

4.8.2.1 Effects on Nrf1 Protein and Gene Expression

Total Nrf1 protein expression did not significantly change in HCT116 cells treated for 4-hours (see Figure 30). Whole cell lysate was used to examine the Nrf1 protein expression, and only showed the total amount of protein present throughout the cells. Future work should include examining the cellular localization of Nrf1 using cellular fractionation techniques to determine whether the treatments induced nuclear localization and Nrf1 signalling within the cells.

No significant changes were found for Nrf1 gene expression with the treatments; however, a non-significant decrease in gene expression was observed with the treatments (see Figure 31).

4.8.2.2 Effects on Nrf2 Protein and Gene Expression

The cellular antioxidant response through Nrf2 was activated and significant nuclear accumulation of Nrf2 protein occurred with 10 and 20 µg/mL PVP-nAg 4-hour treatment in HCT116 cells (see Figure 32). The effect of sodium ascorbate on Nrf2 protein expression was not examined; however, consistent with its role as an important antioxidant, ascorbate has been found to decrease Nrf2 activity in the literature (Tarumoto et al., 2004).

In this work, no significant changes were found in Nrf2 gene expression in the HCT116 cells with 4-hour treatment (see Figure 33). Conflicting results are found in literature, with both increased Nrf2 gene expression with nAg treatment (Aueviriyavit et al., 2014; Gao et al., 2017; Garcia-Reyero et al., 2014) and decreased Nrf2 gene expression being reported (Sun et al., 2017).
4.8.2.3 Luciferase Reporter ARE Activation

Antioxidant response element luciferase reporter assays were used to examine the level of ARE activity resulting from PVP-nAg and AgNO₃ treatments. Nanosilver non-significantly increased the ARE activity through Nrf2 (see Figure 34). However, ARE activity through Nrf1 was significantly decreased with nAg and AgNO₃ treatments (see Figure 35). This indicated that nAg induces activation of the ARE through Nrf2 as opposed to Nrf1.

Reporter gene assays for the Nrf2/ARE pathway have shown an increase in gene activation with nAg treatment in the literature. The N27 neurons transfected with Nrf2/ARE reporter gene resulted in activation of the Nrf2/ARE pathway with nAg treatment as well as increased expression of oxidative stress related genes HO-1 and NQO1 (Chorley et al., 2014). In fact, the Nrf2/ARE pathway has been found to be activated the strongest when compared to nuclear factor kappa B (NF-κB) (involved in the cellular response to various stresses including oxidative stress and the start of inflammation) and activator protein 1 (AP1) (which has a role in various cellular responses including inflammation, cell survival, differentiation, proliferation, and apoptosis) pathways in stable luciferase-reporter HepG2 cell lines (Prasad et al., 2013c).

4.8.2.4 Effects on AhR Protein and Gene Expression

There was no significant change in nuclear AhR protein expression, however, cytosolic AhR significantly increased with 20 µg/mL PVP-nAg treatment (see Figure 36). This increase in cytosolic AhR agrees with the slight decreasing trend in DRE activity with the nAg treatments observed in the DRE luciferase reporter results (see Figure 38). Although no significant change was observed with AhR gene expression (see Figure 37), a decrease in AhR gene expression with nAg treatment has been reported in the literature (Garcia-Reyero et al., 2014).
The AhR transcription factor regulates the cellular response to xenobiotic exposure through activation of the DRE pathway. Once bound to the ligand, AhR translocates to the nucleus and activates the cellular dioxin response through inducing DRE controlled gene expression (Vorrink et al., 2014). The dioxin, TCDD, is used as a positive control for activating the DRE pathway. Compounds that activate the ARE, such as TBHQ and SFN, can also activate the dioxin response through crosstalk with Nrf2 (Shin et al., 2007). The gene for AhR has the ARE in its promoter, allowing the gene expression of AhR to be modulated by Nrf2. Conversely, the gene for Nrf2 has the DRE in its promoter, allowing the gene expression of Nrf2 to be modulated by AhR. Thus, compounds such as TBHQ and SFN that activate Nrf2 may indirectly activate AhR expression (see Figure 36 and Figure 38).

4.8.2.5 Luciferase Reporter DRE Activation

The nAg and AgNO₃ treatments did not induce a significant change in DRE activity, although a slight non-significant decrease is observed. The TCDD positive control significantly increased DRE activity, while TBHQ non-significantly increased DRE activity (see Figure 38). This indicates that the cellular response to nAg involves pathways other than the dioxin response pathway, and the HCT116 cells are mainly responding to nAg treatment through Nrf2 induced activation of the ARE rather than through the DRE (see Figure 32 and Figure 34).

4.8.2.6 Effects on HIF-1α Protein Expression

Nanosilver and AgNO₃ treatment significantly increased HIF-1α protein expression (see Figure 39). Increased HIF-1α expression following nAg treatment has also been reported in the literature (Chen et al., 2020; Jeong et al., 2016; Jung et al., 2014; Manshian et al., 2015).
Combined treatments with both hypoxia and nAg on MCF-7 human breast cancer cells resulted in decreased HIF-1α protein expression compared with cells only treated with hypoxia (Yang et al., 2016). Although not tested in this work, an increase in HIF-1α gene expression with nAg and AgNO₃ treatment has been observed in the literature (Garcia-Reyero et al., 2014).

In this work, treatment with sodium ascorbate both on its own and when combined with nAg, decreased the level of HIF-1α protein present. L-ascorbate is a well-recognized activator of the PHD enzymes involved in the hydroxylation of HIF-1α, leading to the labeling of HIF-1α for proteasomal degradation. L-ascorbate functions as a reducing agent ensuring PHD activity as well as serving as a co-substrate in the active site (Osipyants et al., 2018). Since HIF-1α is involved in the growth and survival of cancer cells in low oxygen environments, treatment with sodium ascorbate is advantageous in decreasing the levels of HIF-1α in cancer cells (Osipyants et al., 2018).

4.9 Nanosilver and ER Stress Response Pathways

Non-toxic nAg and AgNO₃ treatment of HCT116 cells did not induce significant levels of ER stress either through XBP1 or ATF4. Higher AgNO₃ treatments near or above the EC₅₀ value for HCT116 cells induced ER stress, and this was likely involved in cell death.

Evidence of increased ER stress and induced ER stress response through PERK, eIF2-α, IRE-1, ATF-4, ATF-6, and XBP1 have been reported, with this effect depending on the treatment concentrations and cell line used (Huo et al., 2015; L. Li et al., 2018a; Quan et al., 2021; Simard et al., 2015, 2016; Zhang et al., 2012).
4.10 Effects of Nanosilver and Sodium Ascorbate on WNT Signalling Pathways

Very little study has been done on the effects of nAg on the WNT pathways, and no previous research has been found examining the effects of nAg, AgNO₃, and sodium ascorbate treatment on PORCN, WNTless, WNT5A, or WNT3A. Thus, the protein and gene expression of PORCN, WNTless, WNT5A, WNT3A, and β-catenin, were examined in this work.

No significant changes were found in PORCN, WNTless, WNT5A, WNT3A, and β-catenin protein or gene expression with the 4-hour treatments; however, an increasing trend in WNTless protein expression was observed (see Figure 48), and a decreasing trend in WNT5A protein expression was found with sodium ascorbate both on its own and in combination with nAg (see Figure 50). Inhibition of aspects of the WNT pathway is important for cancer treatment (Nygaard et al., 2021), and co-treatment of nAg with sodium ascorbate may be beneficial.

The effect of nAg treatment on β-catenin expression has been examined in a few studies. In addition to its role in the WNT signaling pathway, β-catenin is a cell adhesion protein and has a structural role in which it binds between f-actin and cadherin, assisting in the formation of junctions in the cytoskeleton (Cooper et al., 2019; Natarajan & Kiran, 2019). Treatment of neurospheres with non-toxic levels of 40 nm citrate-stabilized nAg resulted in increased co-localization of β-catenin with f-actin. The level of β-catenin protein was found to slightly decrease in the cells with nAg treatment; however, the cellular distribution and nuclear level of β-catenin did not change, indicating that β-catenin signaling was not activated (Cooper et al., 2019). Similarly, in a wound healing model with zebrafish treated with sodium citrate-stabilized nAg, no change was found in the expression of axin2 and lef1, which are both known to be activated by the Wnt/β-catenin signaling pathway (Pang et al., 2020).
WNT signalling is involved in neuronal development, and the treatment of human embryonic stem cell derived neural progenitor cells with citrate-nAg resulted in increased WNT pathway signaling as determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Li et al., 2021). In zebrafish, β-catenin increased when treated with nAg at concentrations below the EC\textsubscript{50} but resulted in visible morphological abnormalities (Eryılmaz et al., 2018).
Chapter 5: Conclusion

Cell viability studies of PVP-nAg and AgNO$_3$ on HIEC-6 and HCT116 cells demonstrated more toxicity to the HCT116 cells than to the non-cancerous HIEC-6 cells.

Combined treatments of sodium ascorbate with low-dose PVP-nAg showed increased cell death with the HCT116 cells than with the HIEC-6 cells, and was greater than that found with either the nAg or the sodium ascorbate treatments alone. The reverse, however, was not found to be beneficial. In fact, relatively low sodium ascorbate supplementation helped the HCT116 cells to survive the high nAg treatment. Thus, in terms of combined treatments, it was found to be more advantageous to use a higher sodium ascorbate with a low nAg treatment, than to use low sodium ascorbate with high nAg treatment. Future research is needed to determine optimal nAg and sodium ascorbate treatment combinations and concentrations that may be beneficial in cancer treatment, either together or in combination with other drug treatments.

Cell cycle arrest in the G2/M phase was found in the HCT116 cells treated for 24 hours with PVP-nAg. Sodium ascorbate treatment of HCT116 cells at 1 mM did not induce cell cycle arrest on its own. Combined treatment with sodium ascorbate and nAg non-significantly increased the cell cycle arrest from either treatment alone. The cell cycle arrest in the G2/M phase that was seen with the nAg treatment was a silver nanoparticle-specific effect and was not the result of released silver ions. Further research is required to examine the mechanism behind the nAg-induced G2/M cell cycle arrest.

Cellular ROS was not produced in the HIEC-6 or HCT116 cells after 24-hour nAg or AgNO$_3$ treatment in either normoxic or hypoxic conditions with non-toxic treatment concentrations. A shorter treatment time of 4 hours also showed no change in cellular ROS in the
HCT116 cells. The cellular effects seen at these non-toxic nAg treatment concentrations are due to factors other than the generation of oxidative stress.

Mitochondrial oxidative stress was not significantly induced in the HIEC-6 cells, in either normoxic or hypoxic conditions. Significant increases in mitochondrial oxidative stress were induced in the HCT116 cells with 24-hour nAg and AgNO₃ treatment in normoxic conditions. Hypoxic treatment inhibited this induction in mitochondrial oxidative stress. Future research is needed to examine the effect of combined nAg and sodium ascorbate treatments on cytosolic and mitochondrial ROS.

No effect on mitochondrial function and oxidative phosphorylation (as tested with the Seahorse assay) was observed with 4-hour nAg and AgNO₃ treatment in either the HIEC-6 or HCT116 cells; with no significant changes in basal respiration, maximal respiration, ATP production, spare respiratory capacity, non-mitochondrial oxygen consumption, proton leak, or coupling efficiency. Further research would be beneficial to test the effect on mitochondrial function and oxidative phosphorylation of longer treatment times, and to examine the effect of combined nAg and sodium ascorbate treatments.

No significant change in Nrf1 protein expression was observed with the nAg, AgNO₃, TBHQ, SFN, TCDD, hypoxic, and sodium ascorbate treatments. However, a non-significant decrease in Nrf1 gene expression was observed in all the treatments.

The Nrf2 protein expression significantly increased with PVP-nAg in a dose-dependent manner in HCT116 cells. Silver nitrate treatment did not significantly increase the Nrf2 protein expression. Luciferase reporter assays indicated that PVP-nAg treatment induces activation of the ARE detoxification pathway through Nrf2 as opposed to Nrf1. There was a significant increase in cytosolic AhR localization with the 20 µg/mL nAg treatment, which was consistent
with the fact that no activation of the DRE was observed with nAg treatment in the DRE luciferase reporter experiments. It was found that the cellular response to nAg involves pathways other than the dioxin response pathway. Nanosilver and AgNO₃ treatment significantly induced the protein expression of HIF-1α under normoxic conditions in a dose-dependent manner. Additionally, sodium ascorbate treatment both on its own and when combined with nAg non-significantly decreased the HIF-1α protein expression.

No significant induction in ER stress was found with non-toxic PVP-nAg treatment of HCT116 cells. Higher AgNO₃ concentrations significantly induced ER stress through XBP1 and ATF4 in both normoxic and hypoxic conditions.

No significant changes were found in the protein or gene expression of PORCN, WNTless, WNT5A, WNT3A, or β-catenin. Since very little study has been done on the effects of nAg and sodium ascorbate on the WNT pathways, further research is required. Future research is needed in this area to determine whether co-treatment of nAg with sodium ascorbate may be beneficial in decreasing the WNT pathway in cancer, and, if so, to determine optimal treatment combinations and concentrations that may be beneficial. Further investigation of nAg and sodium ascorbate treatment effects on WNT signalling mechanisms, especially through the non-canonical pathway involving WNT5A, should be pursued.

A future avenue of research is to explore the potential beneficial or detrimental effects of nAg and sodium ascorbate on intestinal permeability, inflammation, and leaky gut syndrome.

This work provides a glimpse into the complexities of cellular function and cellular interactions with nAg and sodium ascorbate. The biological interactions of the human body with compounds, or combinations of compounds, are vast and leave much to be still discovered!
Appendices

Appendix A  Optimization Experiments

A.1  Treatment Time Optimization for the Protein Expression of Nrf2 in HCT116 Cells

The peak level of Nrf2 protein expression in HCT116 cells treated with 20 µM TBHQ was found to occur after 4 hours of treatment (see Figure 55). Thus, a 4-hour treatment time was used for the protein expression experiments.
Figure 55: Nuclear and cytosolic Nrf2 protein expression in HCT116 cells treated with 20 μM TBHQ for 0.5, 1, 2, 4, 6, 8, and 24 hours. The values are from one experiment.
A.2 Treatment Time Optimization for Gene Expression in HCT116 Cells

The peak level of Nrf1, Nrf2, AhR, and WNTless gene expression in response to 10 µg/mL nAg treatment in HCT116 cells occurred at 4 hours (see Figure 56). The peak PORCN gene expression occurred around 4 – 6 hours, and the peak WNT5A and WNT3A occurred closer to 6 hours (see Figure 56). Thus, a treatment time of 4 hours was chosen for the qRT-PCR experiments.

Figure 56: Relative Nrf1, Nrf2, AhR, PORCN, WNTless, WNT5A, WNT3A gene expression in HCT116 cells treated at 10 µg/mL PVP-nAg for varying times as analyzed by qRT-PCR. The values are normalized to GAPDH expression. The values are expressed relative to the untreated control and are result of one experiment in duplicate ± S.E.M.
A.3 Optimization of the Transfection Plasmid Amounts

While keeping the overall amount of DNA transfected into the HEK293T cells constant at 10 µg for the same number of cells, increasing the amount of the ARE luciferase reporter pGL4.37 and decreasing the amount of the overexpression plasmid resulted in increased levels of ARE luciferase reporter activity with the same trend being seen in the fold induction (see Figure 57). Thus, transfection amounts of 9 µg of the luciferase reporter with 1 µg of the overexpression plasmid was concluded to be optimal for $1.1 \times 10^6$ HEK293T cells.
**Figure 57:** The ARE Luciferase expression in HEK293T cells with varying transfection amounts of the ARE luciferase reporter pGL4.37 and overexpression of the Nrf2 plasmid shown as (A) fold induction and (B) activity. The values are from 1 to 8 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
A.4  Comparison of the DRE Luciferase activity for the p1A1 and pGudLuc7.5 DRE Luciferase Reporter Plasmids

The two DRE luciferase reporter plasmids had comparable results with the treatments, however, the p1A1 luciferase reporter consistently had slightly higher activity than the pGudLuc7.5 luciferase reporter and was thus used in the experiments (see Figure 58).

**Figure 58:** Luciferase reporter activity from the DRE p1A1 luciferase reporter and pGudLuc7.5 luciferase reporter with pCR3.1 as the empty plasmid control, in response to 24-hour treatment with PVP-nAg, AgNO₃, and TCDD for the control shown as (A) fold induction and (B) activity. The values are expressed relative to the untreated control and are the mean of 3 independent experiments ± S.E.M. Statistical analysis was done using One-Way ANOVA and the significance determined with Dunnett’s test; * p < 0.05, ** p < 0.01, *** p < 0.001.
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