

12-1-2012

Characterization of Beryllium as a Novel Agent to Study Cell Cycle Arrest and Cellular Senescence

Priyatham Gorjala

University of Nevada, Las Vegas, priyathamgorjala@gmail.com

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**CHARACTERIZATION OF BERYLLIUM AS A NOVEL AGENT TO STUDY CELL
CYCLE ARREST AND CELLULAR SENESENCE**

By

Priyatham Gorjala

Bachelor of Science in Bio-Physics, Genetics and Chemistry

Osmania University

2001

Master of Science in Medical Biochemistry,

Manipal Academy of Higher Education

2004

A dissertation submitted in partial fulfillment

of the requirements for the

Doctor of Philosophy in Chemistry

Department of Chemistry

College of Sciences

The Graduate College

University of Nevada, Las Vegas

December 2012



THE GRADUATE COLLEGE

We recommend the dissertation prepared under our supervision by

Priyatham Gorjala

entitled

Characterization of Beryllium as a Novel Agent to Study Cell Cycle Arrest and Cellular Senescence

be accepted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Chemistry

Department of Chemistry

Ronald K. Gary, Ph.D., Committee Chair

Bryan L. Spangelo, Ph.D., Committee Member

Ernesto Abel-Santos, Ph.D., Committee Member

Dong-Chan Lee, Ph.D., Committee Member

Frank Van Breukelen, Ph.D., Graduate College Representative

Tom Piechota, Ph.D., Interim Vice President for Research &
Dean of the Graduate College

December 2012

ABSTRACT

Characterization of Beryllium as a Novel Agent to Study Cell Cycle Arrest and Cellular Senescence

By

Priyatham Gorjala

Dr. Ronald K. Gary, Examination Committee Chair

Associate Professor, Biochemistry

Department of Chemistry

University of Nevada, Las Vegas

Cancer cells evade senescence, apoptosis, and other constraints on proliferation, often via mutation of the p53 tumor suppressor gene (*TP53*). Normal human lung fibroblasts have been shown to enter premature senescence upon exposure to beryllium. In these cells, BeSO₄ stabilizes p53 protein, increases p21 gene expression, induces senescence-associated β-galactosidase activity and causes cell proliferation arrest. In the present study, we have investigated whether BeSO₄ is able to induce similar effects in cancer cells that have wildtype p53. We have demonstrated that beryllium salt at low concentration can induce molecular changes in the p53 signaling pathway leading to cell cycle arrest in beryllium-sensitive cancer cells. The p53 transcription factor is the central regulator of the mammalian DNA damage response. However, unlike most agents that activate p53, beryllium did not cause DNA damage. We have discovered that the growth arresting effects of beryllium are mediated through the p53-dependent p21 pathway. We have also found that BeSO₄ may be influencing the regulation of cyclin E2, a cyclin that controls passage of cells from G1 to S phase of the

cell cycle. Increased caspase 3/7 activity was also seen in response to BeSO₄ treatment. However, the number of cells undergoing apoptosis was not proportional to the activity of caspase 3/7, suggesting that the beryllium response is primarily cytostatic rather than apoptotic. These observations indicate that beryllium suppresses cell proliferation through a senescence-related p53 signaling pathway that differs from the DNA damage response pathway. These unusual properties make beryllium salt a potent tool to investigate p53-mediated growth control pathways in both normal and cancerous cell types.

ACKNOWLEDGEMENTS

During the last 6 years I have spent in Las Vegas, many people contributed in small and big ways that have ultimately allowed to me to successfully complete the Ph.D. program. I would like to thank all those people who have helped me through this journey.

First of all I am profoundly indebted to my advisor Dr. Ronald K Gary, a true personification of mentorship. He has been very generous with his time from day 1. Through countless hours spent in long meetings he has inculcated and honed scientific thinking in me. He has also taught me good laboratory methods and nuances that would be otherwise difficult to learn. I always left our meetings with a renewed motivation, confidence and direction.

I would like to thank my committee members, Drs. Bryan L. Spangelo, Ernesto Abel-Santos, Dong-Chan Lee and Frank van Breukelen for their support and feedback. I also would like to thank Dr. Dennis W. Lindle (Chair, Dept of chemistry), Dr. Kathleen A. Robins (Graduate Coordinator, Dept of chemistry) for supporting me with Teaching Assistantship.

I want to express my gratitude to Ms. Swapna Mudireddy and Mr. Ata ur Rahman Mohammed Abdul for all the help in the laboratory work and for being great colleagues. I would like to thank Ms. Casey Hall and Ms. Shirley Shen at the UNLV Genomics Core lab for their professional help.

I greatly appreciate my friends Drs. Vamsee Krishna GK, Raja Ram Chamala, Ramesh Boggula, Venkateshwar Madka and Mr. Nageswara Rao, for their unlimited support and encouragement whenever I needed it. I would like to thank Dr. Mukund, Mr. Balaji, Mr. Sharath, Mr. Suman, and Ms. Pallavi for helping me settle down in Las Vegas and making my time here a memorable experience.

Finally, I could not have done this without my family. I cannot thank my mom, dad and brother enough for their unconditional love and support. I feel so fortunate to have my wife Padma with me to go through this journey together.

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CHAPTER 1 INTRODUCTION

PURPOSE OF STUDY

Beryllium (Be^{2+}) is a divalent cation that has the ability to inhibit growth of various cell lines. The exact mechanism of action as to how beryllium causes cell cycle arrest or shows anti-proliferative effect is not clearly understood. It has been demonstrated that Be^{2+} in extremely low doses over short duration increases p21 mRNA and in long exposures can induce expression of senescence-associated β -galactosidase activity in Human fibroblasts (HFL-1) (Coates et al., 2007). The aim of this study is to improve our understanding of the mechanism of action of beryllium salt as a cytostatic agent.

- Chapter 3 presents the results of a survey on the effect of beryllium on various cancer cell lines and identifies a beryllium sensitive and a resistant cell line for further study.
- Chapter 4 covers the data from a study that was done to evaluate the effect of beryllium on susceptible cancer cell line. Beryllium was found to be able to induce senescence-like cell cycle arrest through a pathway that may be different from DNA damage pathway.
- Chapter 5 includes data that demonstrates p53 dependence of the effects of beryllium especially p21 increase and cell cycle arrest. The experiments were done using A172 cell line (human glioblastoma) with p53 expression or function knocked down using 3 different techniques (i.e., siRNA, shRNA and E6 protein expression.)
- Chapter 6 documents the results of experiments done to analyze the level of caspase 3/7 activation and to verify if the activation of caspase 3/7 results in apoptosis.

RESEARCH QUESTIONS

Beryllium can induce growth arrest in different types of cell lines. The existing evidence indicates that it may be using p53-p21 pathway to achieve this. In this thesis we have attempted to answer the following research questions:

1. Research question 1: Does beryllium induce growth arrest equally in various cell types?

Test: Survey 12 cell lines exposed to eight different concentrations of beryllium ranging from 0-1000 μ M over a period of 5 days and count the total number of attached cells present at the end of growth period using Cy-Quant assay.

2. Research question 2: Does beryllium dosing induce expression of p21 and senescence in cancer cell lines similar to HFL-1 cells?

Test: Using a beryllium sensitive and a resistant cell line as representatives of the respective groups investigate the changes in expression of p21 mRNA and protein levels, measure the amount of beryllium uptake by using ICP-MS, quantify the expression of SA β gal.

3. Research question 3: Is p21 induction and cell cycle arrest induced by beryllium a p53 dependent change?

Test: Knock down p53 expression using siRNA, shRNA and E6 protein expression. Use the resulting cell types to study the short term and long term changes in molecular events like p21 upregulation and other cell cycle regulator proteins and gene expression.

4. Research question 4: Does beryllium induce apoptosis?

Test: Measure the activation of caspase 3/7 in cells exposed to beryllium and evaluate the extent of apoptosis using TUNEL assay.

CHAPTER 2 REVIEW OF LITERATURE

BERYLLIUM

Beryllium was discovered by Nicholas-Louis Vauquelin in the year 1797. It was first separated in 1828 independently by a French chemist AntoineAlexandre-Brutus Bussy and German chemist Friedrich Wöhler (De Laeter et al., 2003).

Beryllium (Be) is an alkaline earth metal with atomic number 4. It belongs to Group IIA of the periodic table and has two oxidation states, Be(0) and Be(+2). Due to high reactivity of beryllium it is available in approximately 45 mineral forms of which Beryllium aluminium silicate (beryl), $3\text{BeO}\cdot\text{Al}_2\text{O}_3\cdot 6\text{SiO}_2$ and Bertrandite ($4\text{BeO}\cdot 2\text{SiO}_2\cdot\text{H}_2\text{O}$) are the two commercially important ones.

Name	Chemical Formula	Molecular Weight	Physical State	Solubility in Water
Beryllium	Be	9.012	Solid, hexagonal structure	Insoluble
Beryllium fluoride	BeF_2	47.01	Glassy, hygroscopic mass	Very soluble
Beryllium chloride	BeCl_2	79.92	Needles	Very soluble
Beryllium hydroxide	$\text{Be}(\text{OH})_2$	43.03	Amorphous powder or crystalline solid	Sparingly soluble
Beryllium oxide	BeO	25.01	Amorphous powder	Very sparingly soluble
Beryllium phosphate	$\text{Be}_3(\text{PO}_4)_2$	271.03	Crystals	Soluble
Beryllium sulfate **	$\text{BeSO}_4\cdot 4\text{H}_2\text{O}$	177.13	Tetragonal crystals	Soluble

Table 2-1 Properties of beryllium compounds

Beryllium is one of the lightest metals. It has a high charge-to-radius ratio and forms covalent bonds. Physical properties and solubility of beryllium compounds are summarized in Table 2.1. There are nine known radioisotopes of beryllium of which beryllium-7 (Be-7) and beryllium-10 (Be-10) are stable with half lives of 53.29 days and 1.51×10^6 years (C. R. Hammond, 2011-2012).

The very first observation of the ability of beryllium to stop cellular growth was documented in 1949, using an animal limb regeneration model (Chèvremont and Firket, 1951; Thornton, 1949). Two years later, the first anti-mitotic effects in a cultured cell system were reported in *Nature* (Chèvremont and Firket, 1951). Historically most of the studies on beryllium and its effects on cells have been done with an objective to evaluate the toxicity of the molecule and diseases associated with chronic exposure to it (Reeves et al., 1967; Schepers, 1961). Typically, such toxicity studies employ metallic beryllium, or beryllium salts at very high concentrations. In contrast, only a few studies have been done to understand the mechanism of action of low dose beryllium salt as a cell proliferation inhibitor (Skilleter et al., 1983; Skilleter and Paine, 1979; Williams and Skilleter, 1983). In our research we have attempted to study the effect of beryllium on cancer cells. For experiments we have used beryllium sulphate tetrahydrate, a soluble form of beryllium. We have used beryllium at very low concentration (approximately 10 μM) in short time points between 2-7 days. Our research focus was on the effect of beryllium at low dose, short time point exposures resulting in specific responses in the form of gene expression, cell cycle arrest etc. We have discovered that beryllium, just like traditional chemotherapy drugs or radiation, can bring about cell proliferation arrest or increase in p53 and p21 proteins. However, unlike the actions of chemotherapeutics and radiation, these responses due to beryllium are stronger and without DNA damage (Gorjala and Gary, 2010).

p53

Two different types of studies, one using virologic approach and the other with a serologic approach led to the discovery of p53 in the year 1979 (DeLeo et al., 1979; Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Rotter et al., 1980). Observations made immediately after discovery of p53 suggested that it may be anti-oncogenic. Data from research in the subsequent years showed that wild type p53 genes were growth suppressive and several cancer cell lines had mutations in p53 gene. For example HeLa cells expressed normal levels of p53 mRNA but undetectable levels of protein (Crawford et al., 1981). Human promyelocytic leukemia cells, HL-60, has p53 gene with large deletions that results in no p53 protein expression (Wolf and Rotter, 1985). These discoveries led to identification of anti-oncogenic and normal function of p53.

The p53 gene (called *TP53*) is located on the short arm of chromosome 17 (17p13) (Isobe et al., 1986). The gene is 22,000 bp long with 11 exons while its mRNA is 2.2 kb (Lamb and Crawford, 1986). p53 is a 393 aminoacid protein with three distinguishable domains. The transactivation domain is located at the amino terminus and is targeted by proteins to regulate p53's function as transcription factor. The DNA binding region of p53 is located in the central part of the protein. DNA binding region is followed by an oligomerization domain at carboxy terminal region that mediates tetramerization of p53. The C-terminal region of the protein contains a negative regulatory domain as well as lysine residues that are involved in targeting the protein to ubiquitin based degradation by proteosome.

In recognition of the significant role of p53 protein in monitoring the integrity of genome it has been called the "Guardian of the Genome" (Lane, 1992). Under normal conditions the half life of p53 is between 5-30 min. When the cells are exposed to genotoxic stress stimuli, cellular levels of p53 protein are found to increase by rapid

stabilization. Many post translational modifications stabilize the protein and prevent it from degradation. p53 protein activates DNA repair mechanisms, induces or inhibits expression of many target genes (El-Deiry, 1998). p21 mRNA, expressed in a p53 dependent as well as independent manner, transcribes a cyclin dependent kinase (CDK) inhibitor which blocks cell cycle progression.

Depending on the kind and extent of damage induced by the stress, p53 protein can induce cell cycle arrest or trigger apoptosis. The decision between cell cycle arrest or apoptosis depends on total amount of p53 in cells, extent of DNA damage, sub cellular localization and other factors like post translational modifications of the protein (Chen et al., 1996; Lassus et al., 1996; Moll et al., 1996; Ostermeyer et al., 1996). While cell cycle arrest gives cells an opportunity to overcome damage and survive, apoptosis kills the cells thus preventing abnormal cells from surviving. Because p53 causes two extreme outcomes for cells it is placed under tight regulation with a very short half-life.

CELL CYCLE ARREST, SENESENCE AND APOPTOSIS

Cell cycle arrest

A series of phases that an eukaryotic cell crosses before it divides into two daughter cells, are together termed as cell cycle. Cell cycle is composed of Gap before DNA synthesis (G_1), DNA synthesis (S), Gap after DNA synthesis (G_2) and mitosis (M). Cells have check points during cell cycle to maintain the genomic stability. These checkpoints ensure that all the events specific to each phase materialize before the cell moves into the next phase of cell cycle (Hartwell and Weinert, 1989). Cell cycle may be arrested at these checkpoints if cell does not have enough supply of growth factors or has not repaired any cellular damage that might have occurred. Cell cycle check points can also activate apoptosis pathway that results in removal of cells that may become potentially tumorigenic due to failed genetic repair.

The first cell cycle check point is at the G₁-S transition. This check point is also the most extensively studied. The cell cycle arrest at G₁-S check point is initiated in response to DNA damage. This check point provides enough time to the cell to repair the damage and allows it to make a decision about dividing or delaying the division or entering a G₀ resting phase. During DNA damage the stabilized p53 protein introduces G₁-S arrest (Baker et al., 1990; Diller et al., 1990). p53 causes this arrest by transcriptionally increasing the Cdk inhibitor p21 (El-Deiry et al., 1993).

Beryllium was shown to induce G₁ cell cycle arrest in rat liver cells (Skilleter et al., 1983). p53 and p21 were found to be increased by beryllium in human skin and lung fibroblast cells (Lehnert et al., 2001; Coates et al., 2007). There were no studies so far on the effect of beryllium on the cell cycle of cancer cell lines, until our work described in Chapter 4 and published in 2010 (Gorjala and Gary, 2010).

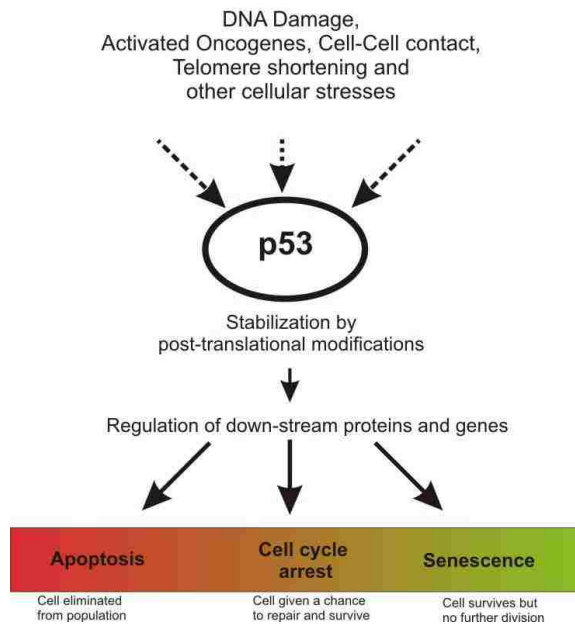


Figure 2-1 Overview of effects of p53

Senescence

Cultured primary cells cease to proliferate after a specific number of divisions. This property called as replicative senescence is natural to primary cells in culture and

was observed in human fibroblast cells by Hayflick and Moorhead (Hayflick and Moorhead, 1961). Senescence is different from cell growth arrest both physiologically and morphologically. Senescence is triggered by telomere shortening that accrues with successive rounds of cell division. Activation of p53 and pRB/p16INK4a pathways is required for induction of senescence in human cell lines (Ben-Porath and Weinberg, 2005; Shay, 1991). Senescence-associated β -Galactosidase activity (SA- β -gal) is an important enzymological biomarker for senescence (Dimri et al., 1995).

A study from our lab group showed that beryllium, at very low doses, can cause dose dependent induction of premature senescence in human fibroblasts (Coates et al., 2007). The study has shown that beryllium causes sustained increase of p53 protein and p21 mRNA and protein on short term exposure. Increase of p16^{Ink4a} and Senescence-associated β -Galactosidase activity were seen during long term exposures. Recent studies suggest that activation of p53 in tumor cells leads to tumor regression by induction of senescence (Ventura et al., 2007; Xue et al., 2007).

In present study (Chapter 4) we have data that shows that beryllium causes cell cycle arrest and brings about senescence-like state in A172 glioblastoma cancer cell line at a low dose of 10 μ M. In subsequent work, we found that these cell cycle effects of Be²⁺ are p53-dependent (Chapter 5).

Apoptosis

The term apoptosis first proposed by Kerr et al., to describe a form of controlled “cell deletion” (Kerr et al., 1972). Since its discovery apoptosis or programmed cell death has been recognized as a central process required for normal development of an organism and also to maintain homeostasis (Lockshin and Williams, 1965; Jacobson et al., 1997; Danial and Korsmeyer, 2004). Apoptosis involves a series of energy dependent molecular events that result in various morphological changes that are observed and documented using light and electron microscopy (Hacker, 2000). Two

major pathways induce and execute apoptosis: (1) extrinsic or death receptor pathway and (2) intrinsic or mitochondrial pathway. In addition, there is also a recently discovered T-cell mediated cytotoxicity and perforin-granzyme-dependent pathway. Caspases also called as executioners of apoptosis are cysteine proteolytic enzymes with specificity to aspartic acid residues. Activated caspases cut their targets next to aspartic acid residues and can in turn activate other caspases through a cascade pathway. They disrupt important pathways by targeting protein kinases, DNA repair and structural proteins of the cells ultimately leading to controlled cell death (Cohen, 1997; Nicholson, 1999). Caspase activity in the cell is tightly regulated. They are synthesized as inactive zymogens and require to be activated to become functional. Inhibitors of apoptosis (IAP's) are a family of proteins that can inhibit activity of caspases to rescue the cell from apoptosis (Fuentes-Prior and Salvesen, 2004).

Evidence from many studies during early 1990's showed that p53 could control apoptosis (Yonish-Rouach et al., 1991; Clarke et al., 1993; Lowe et al., 1993; Symonds et al., 1994). However, the exact mechanism as to how p53 chooses between cell cycle arrest, senescence and apoptosis is not yet clear. p53 is known to transcriptionally activate the proapoptotic genes Bax, Puma, Noxa and Bid (Miyashita et al.; Oda et al., 2000; 1994; Nakano and Vousden, 2001; Sax et al., 2002). Apart from controlling expression of these genes, p53 is also known to regulate components of intrinsic and extrinsic pathways of apoptosis (Owen-Schaub et al., 1995; Muller et al., 1998; Kannan et al., 2001; MacLachlan and El-Deiry, 2002).

Our published experimental data showed increase of p53 in beryllium treatment, so we have used caspase and TUNEL assays to evaluate whether apoptosis is induced by beryllium treatment (Chapter 6).

CHAPTER 3 BERYLLIUM SENSITIVITY OF CANCER CELL LINES - CELL LINE SURVEY

INTRODUCTION

Earlier studies have documented the effects of beryllium on normal cells. One of the very early studies of effects of beryllium used time-lapse cinematographic analysis and found that beryllium increased the division time of human lung fibroblasts (HFL-1) (Absher et al., 1983). Many studies around this period and earlier have linked beryllium to two diametrically opposed effects: carcinogenesis and growth inhibition. These studies have used various cell types including HFL-1, rat liver cells, skeletal muscle cells, macrophages etc, and various salts of beryllium and concentration ranges. A list of these studies can be found in paper by Wagoner et al (Wagoner et al., 1980). A study by Hart et al (Hart et al., 1982) has documented the effect of beryllium in various forms on growth of human lung fibroblasts. A recent study by Coates, S.S et.al has attempted to systematically investigate the mechanism of action of beryllium. The study demonstrates that beryllium has anti-proliferative effect on normal human lung fibroblasts (Coates et al., 2007). The study also shows that there is an interaction between promoter of p21 gene and p53 protein that results in an increase of p21 gene. In response to beryllium treatment the cells exhibit G1 to S phase block leading to G1 arrest.

The evidence that beryllium causes increase of p53 and p21 proteins and also growth arrest at a very low concentration of 10 μ M gave us the motivation to study the effect of beryllium on cancer cell lines. Our expectation is that beryllium may be able to cause a similar increase of p53 and p21 leading to growth arrest in cancer cell lines that have functional p53 and p21 genes. The response of cancer cell lines to a drug will be different due to the variation in genetic and phenotypic makeup of the cells. With this idea we have conducted a survey of responses of 12 cell lines to a range of concentrations of beryllium. The objective of this survey study is to verify if different cell

lines respond differently to beryllium dose and to select a representative cell line for sensitive and resistant varieties.

MATERIALS AND METHODS

Cell culture media

RPMI glutamax-1 + 25mM HEPES Cat# 72400, PSF – Gibco antibiotic and antimycotic Cat# 15240-062, 0.05% Trypsin-EDTA - Cat# 25300 are purchased from Invitrogen - Gibco (Carlsbad, CA). Fetal Bovine serum - Cat# SH30071 and phosphate buffered saline – Cat# ARC25970 are from Hyclone Laboratories (Logan, UT). Cyquant cell proliferation kit was obtained from Molecular probes.

S91 cell line requires F-12K Medium (Kaighn's Modification of Ham's F-12 Medium). S91 cells from stock were thawed into F12K medium supplemented with 15% calf serum, 2.5% fetal bovine serum and 1x antibiotic and antimycotic. The cells were slowly adapted to regular RPMI medium mentioned above.

Cell lines

A172,BT4C, C6, U87MG, PC12, U251, HeLa, PC3, S91 and RKO were purchased from ATCC (Manassas, VA). Cell lines SF539, SNB75 were purchased from National Cancer Institute. Cells were cultured in RPMI 1640 with Glutamax and 25mM HEPES supplemented in 10% FBS and 1%PSF.

Cell culture and CyQuant assay

Cells from 100mm plates were trypsinized using 1mL of 0.05% Trypsin-EDTA followed by incubation for 3 minutes at 37°C and 5%CO₂. The cells were then resuspended in 7 mL of fresh cell culture media. The number of cells was calculated using coulter counter and reseeded as required.

Each cell line used in the experiment was seeded to 60 mm plates and allowed to reach 100% confluency. The cells in 60 mm plates were trypsinized by adding 500 µL of 0.05% Trypsin-EDTA and incubated at 37°C and 5% CO₂ for 3 minutes. The cells

were resuspended in 2.5 mL RPMI. 100 μ L of cell suspension was mixed with 9.9 mL of Isoton solution and used for taking cell counts in coulter counter. This number is used to determine the number of cells required for each cell line to reach 100% confluency.

Day -1, Seeding to 60 mm plates: The cells from 100 mm plates were trypsinized as described before and available number of cells were determined by using coulter counter. For each cell type the required number of cells was calculated and twenty four 60 mm plates were seeded (8 doses x 3 replicates = 24).

Day 0, Beryllium dosing: RPMI is prepared with 0, 1, 3, 10, 30, 100, 300 and 1000 μ M BeSO₄. 10 mL of RPMI at each concentration of BeSO₄ is prepared for each cell line. The plates seeded on day -1 are screened for uniform distribution and confluency. Plates are shuffled, labeled and media is replaced with freshly prepared beryllium RPMI corresponding to the labels. Adding beryllium RPMI marks the beginning of time point and the day is counted as Day 0.

Day 3, Transfer to 96 well plate: 60 mm plates were screened to check for confluency. The control (0 μ M beryllium RPMI) plates were trypsinized as described before and cell counts determined using coulter counter. Volume of cell suspension required to seed 16000 cells per well is calculated and mixed with RPMI to get final volume to 200 μ L. The volume calculated for control plates is used for all concentrations of BeSO₄. Using the volume calculated for control cells enabled us to transfer the number of cells proportional to the ones remaining in the plates after experiencing the effect of beryllium. Cells from each 60 mm plate were seeded into 3 replicate wells on the 96 well plate. The concentration of BeSO₄ was maintained during the transfer of cells from 60 mm to 96 well plate.

Day 5, Standard curve seeding and termination: Approximately 8 hours before terminating the experiment, standard curve seeding is done. Untreated cells for each cell line are trypsinized from a 100 mm plate and resuspended in RPMI. Cell counts are

taken and dilutions are prepared to give 62500, 31250, 15625, 7813, 3906, 1953, 976 and 0 cells per well. The diluted cells are seeded to three replicate wells.

Experiment was terminated 8 hours after the standard curve cells were seeded. To terminate the experiment the plates were removed from incubator and inverted in sink to remove RPMI. 200 μ L of PBS is added to wash the cells in all wells. This step is done carefully taking precaution not to dislodge any cells. PBS is removed by inverting the plate in sink and blotting over stack of paper towels. The 96 well plate is wrapped in aluminum foil and saved at -80°C till further processing.

CyQuant assay: On the day of assay the plates are removed from -80°C and placed on dry ice for 3 hours, contributing to a freeze-thaw cell lysis step. The plate is allowed to thaw to room temperature and 200 μ L of CyQuant solution, prepared by mixing 19 mL Millipore water, 1 mL CyQuant cell lysis solution and 50 μ L CyQuant GR dye, is added. The plate is wrapped in aluminum foil and incubated at room temperature for 5 minutes and readings are taken on Genios plate reader set to 486 nm and 535 nm of excitation and emission wavelengths respectively.

Data analysis

The fluorescence reads from wells with standard curve were plotted on y-axis with corresponding cell number on x-axis. A linear regression trend line was added and the slope in resulting equation was used to calculate the cell number in each well. Average of replicates is plotted for all cell lines.

RESULTS

Beryllium in the form of $\text{BeSO}_4 \cdot 4\text{H}_2\text{O}$ was diluted from primary stock to give final working concentrations of 0, 1, 3, 10, 30, 100, 300 and 1000 μM . The cells were cultured in the presence of beryllium in 60 mm plates for 3 days and then reseeded to 96 well plate as described earlier. At the end of 5 days under beryllium influence, the total DNA content of cells in each of the wells of the 96 well plate was calculated using Cy-Quant

assay, which employs the dsDNA dye CyQuant. The volume of cell suspension transferred from 60 mm plates to the wells of 96 well plate is same for cells at all concentrations of BeSO₄. This ensured that the number of cells transferred to 96-well plate are proportional to the number of cells remaining attached to the 60 mm plate at the end of day 3. The total period of exposure to the drug was for 5 days. The concentration of BeSO₄ and the proportion of number of cells attached to 60mm plates are maintained during the transfer of cells from 60 mm plates to the wells of 96 well plate. This assay measures the differential growth of treated cells during the 5 day treatment period.

The results shown in Fig 3.1 and 3.2 demonstrate that all cancer cells are not sensitive to beryllium. Out of 12 cell lines used for this survey 6 cell lines were considered sensitive and 6 cell lines were resistant. The classification was done based on IC50 values of beryllium for each cell line. The IC50 value represents the concentration of drug required to produce 50% inhibition of cell number (as judged by total DNA content) during the growth period in the 96-well plates. A172, a human glioblastoma cell line and SF539, a glioma cell line from human, were the most sensitive of all the cell lines. RKO, a human colon carcinoma and PC-12, a pheochromocytoma from rat were the most resistant.

DISCUSSION

The green fluorescent dye in the Cy-Quant reagent binds to the DNA in cells. The amount of fluorescence is directly proportional to the number of cells in the wells. Cells were serially diluted and seeded to generate values for standard curve. The equation from standard curve is used to calculate the number of cells in control and beryllium dosed wells.

Beryllium Sensitive Cancer Cell lines

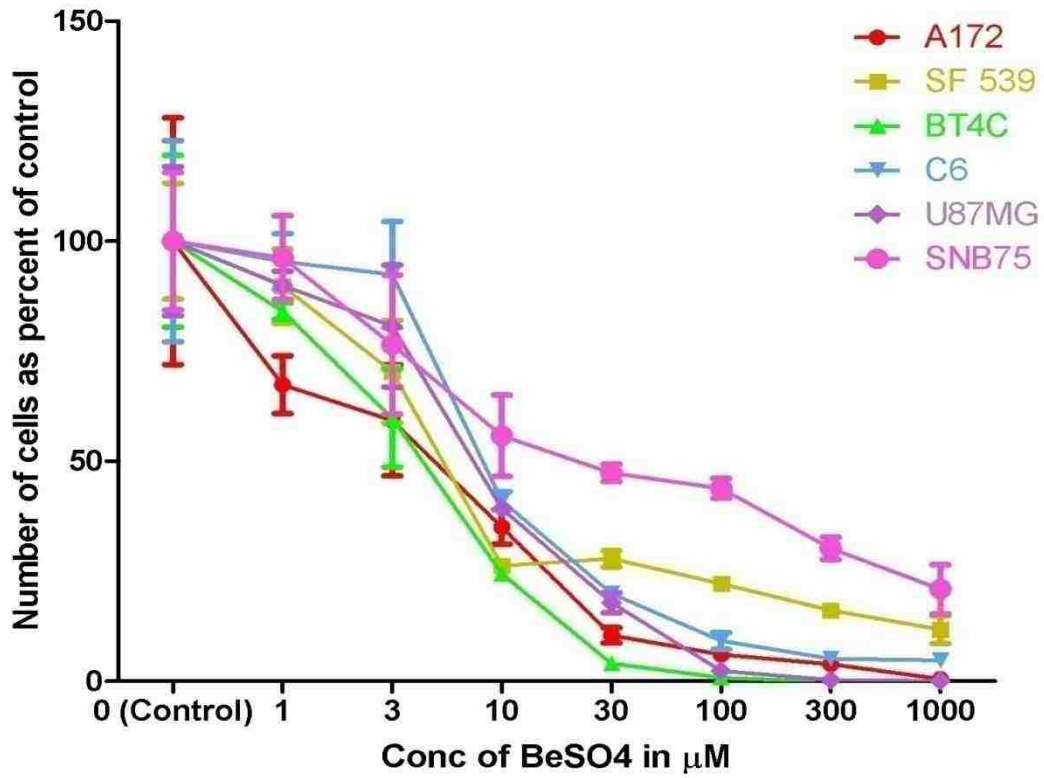


Figure 3-1 Beryllium sensitive cell lines

A172 (human, glioblastoma) and SF539 (human, glioma) were the most sensitive cell lines. Both of these cell lines showed highest sensitivity to beryllium by showing decrease in cell number even with IC50 values of 3.4 and 5.1 μM respectively for beryllium. The data are presented as mean \pm Standard Deviation.

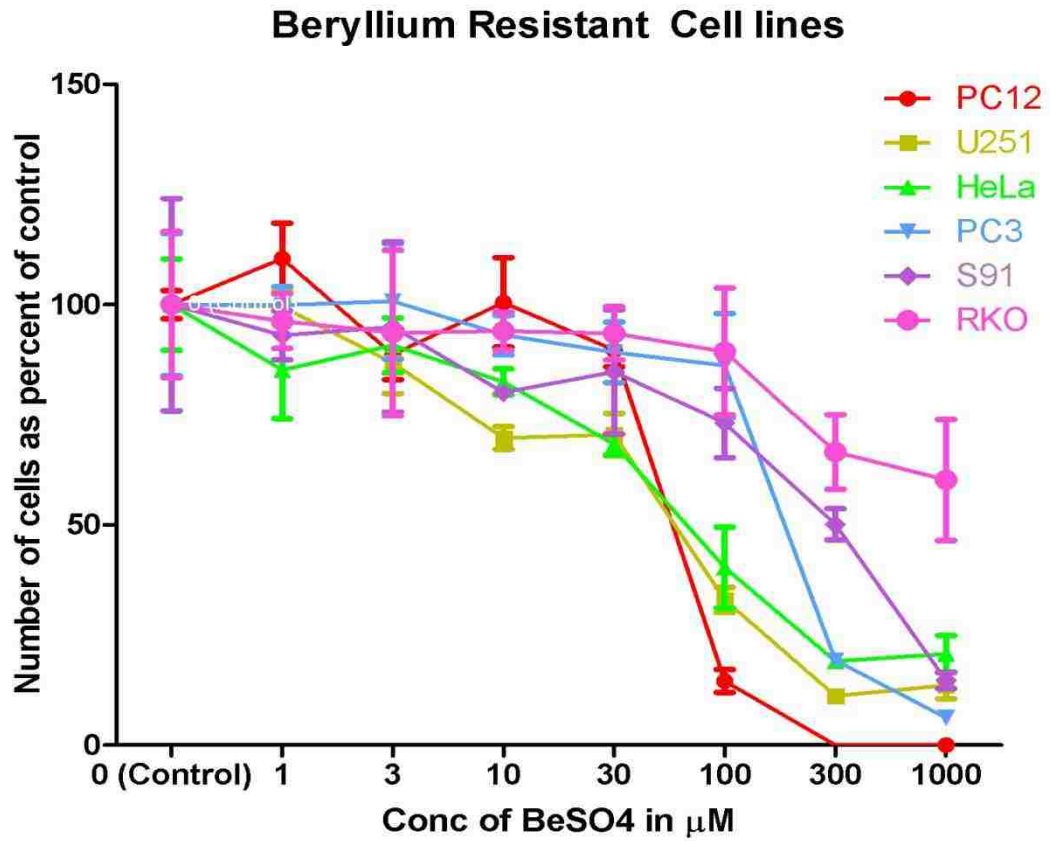


Figure 3-2 Beryllium resistant cell lines

RKO (human, colon carcinoma) and S91 (pheochromocytoma, rat) were the most resistant cell lines. Both of these cell lines showed resistance and ability to survive presence of beryllium with IC₅₀ values of 440 and 290 μM of BeSO₄. The data are presented as mean +/- Standard Deviation.

Role of p53 in the cellular response to the treatment of cells with beryllium has been documented in earlier studies (Gary and Kindell, 2005; Lehnert et al., 2001). Table 3.2 documents the p53 status of the different cell lines used in this survey. It is possible that the difference in the status of p53 might have contributed to the variation in response of cell lines to beryllium treatment. However, with the data presented here, there is no correlation between the p53 status and beryllium resistance. A detailed study is required to thoroughly understand the mechanism of action and also to explain the differences in response to beryllium.

For further study about the mechanism of action of beryllium, we chose to use human cell lines A172 and RKO as representatives of sensitive and resistant cell lines, respectively. The choice was based on the IC50 values listed in Table 3.1. Both these cell lines express functional p53 protein (Mirzayans et al., 2005; Ishii et al., 2006; Liu and Bodmer, 2006).

The data presented in later chapters show that some of the effects of beryllium include cell cycle arrest and p53 dependent induction of p21. It has been established in earlier studies that p53 protein plays an important role in cell cycle arrest and cell lines having mutated p53 or lacking p53 may show some resistance to action of cytotoxic drugs (Amundson et al., 1998; Shaw, 1996; Brosh and Rotter, 2009).

S.No	Cell Line	IC 50 Value (μM of beryllium)
1	A172	3.4
2	SF539	5.1
3	BT4C	8.5
4	C6	8.9
5	U87MG	12
6	SNB75	22
7	PC12	56
8	U251	59
9	HeLa	72
10	PC3	180
11	S91	290
12	RKO	440

Table 3-1 IC50 values of BeSO₄ for different cell lines

Different cell lines have different levels of sensitivity to beryllium treatment. Some cell lines can tolerate very high concentration of beryllium while some are sensitive to its presence even in very small concentrations. The IC₅₀ values are calculated from the data used to plot Fig 3.1 and 3.2

S.No	Cell Line	Type, Origin	Species	ATCC Cat #	TP53 status	References
1	A172	Glioma, Brain	<i>Homo sapiens</i>	CRL- 1620	Wild Type	(Mirzayans et al., 2005; Ishii et al., 2006)
2	SF539	Glioma, Brain	<i>Homo sapiens</i>	NCI-60 cell line *	Single mutation, Frame shift mutation at position 342 deletion of 1 A	(Forbes et al., 2010)
3	BT4C	Glioma, Brain	<i>Rattus norvegicus</i>			
4	C6	Glioma, Brain	<i>Rattus norvegicus</i>	CCL-107	Wild Type	(Asai et al., 1994)
5	U87MG	Glioma, Brain	<i>Homo sapiens</i>	HTB-14	Wild Type, Homozygous	(Ishii et al., 2006; Van Meir et al., 1994)
6	SNB75			NCI-60 cell line *	Mutant p53, position 258 G to A	(O'Connor et al., 1997)
7	PC12	Pheochromocytoma	<i>Rattus norvegicus</i>	CRL-1721	Functional p53 protein expressed	(Eizenberg et al., 1996)
8	U251	Glioma, Brain	<i>Homo sapiens</i>		Mutant p53, position 273 R to H	(Brazdova et al., 2009)
9	HeLa	Adenocarcinoma, Cervix	<i>Homo sapiens</i>	CCL-2	p53 mRNA expressed but protein is degraded due to complexing with HPV proteins	(Scheffner et al., 1991)
10	PC3	Adenocarcinoma; Metastasis to bone, Prostate	<i>Homo sapiens</i>	CRL-1435	No p53 mRNA. Single copy of p53 gene has deletion at codon 138 resulting frame shift placed a stop codon at 169.	(Carroll et al., 1993; Isaacs et al., 1991)
11	S91	Melanoma, Skin	<i>Mus musculus</i>	CCL-53.1		No ref available
12	RKO	Carcinoma, Colon	<i>Homo sapiens</i>	CRL-2577	Wild type p53	(Liu and Bodmer, 2006)

Table 3-2 p53 status of different cell lines used in the survey

p53 plays a significant role in the effectiveness of drugs that interfere with cell cycle controlling pathways.

CHAPTER 4 BERYLLIUM INDUCES SENESCENCE LIKE STATE IN GENETICALLY SENSITIVE TUMOR CELL LINES – COMPARATIVE STUDY BETWEEN A172 AND RKO *

INTRODUCTION

Normal somatic cells undergo cell division, after a specific number of divisions they enter a phase called cellular senescence. Senescence is characterized by very long population doubling times and changes in morphology of cells however, the cells are metabolically active (Campisi, J.,2001). Replicative senescence is the name given to the process that is a result of decreased ability of normal cells to divide due to shortening of telomeres after multiple cell divisions (von Zglinicki, 2001). Senescence induced by factors other than telomere shortening is known as premature senescence or accelerated senescence. Senescence in general is believed to be a tumor suppressor mechanism developed by cells. When normal cells undergo mutations that give them ability to override the senescence mechanism they turn into cancer causing cells.

It is now established that some types of tumor cells can be made to become senescent by exposing them to drugs, radiation or genetic manipulation. Some of the therapeutic agents that are used in treatment of cancer function like senescence inducing agents. These drugs inflict DNA damage which in turn activates the p53 dependent senescence pathways *in vitro* (Chang, B. D., 1999). p53 is at a pivotal point

* NOTE: This chapter corresponds to the content of the published paper: Gorjala and Gary (2010). Beryllium sulfate induces p21^{CDKN1A} expression and a senescence-like cell cycle arrest in susceptible cancer cell types. *Biometals* 23: 1061-1073.

Two versions of this manuscript were written independently. The version of the manuscript as it appears in *Biometals* was written by Dr. Ronald K. Gary. The alternate version of the manuscript, which appears here as Chapter 4, was written by Priyatham Gorjala.

in intracellular signaling network that detects DNA damage and controls the progression of cell cycle (Di Leonardo, A. et al, 1994). The p53 regulated cell cycle arrest can be initiated by various means that include DNA damage, oxidative stress, HDAC inhibitors etc (Drayton S et al 2002). p53 and p21 play a key role in induction of senescence in normal cells and are also responsible for senescence in human tumor cells treated with therapeutic drugs (Chang, B. D., 1999). p53 senses DNA damage due various stress stimuli, short telomeres or activated oncogenes. The activated p53 mediates its cell cycle arresting actions through p21. p21, a cyclin dependent kinase inhibitor, is famous for its role in cell cycle arresting abilities. Many studies have proved that p21 causes G1 phase arrest in cell cycle. G1 phase arrest occurs as a result of cyclin dependent kinase inhibition in the initial phases of senescence and the changes are coordinated by action of p21 during the early phases of senescence (Dulić V et al., 2000).

Induction of cellular senescence pushes cells from actively proliferating state to a non-proliferating senescent state. Induction of cellular senescence in tumor cells is being considered as well as researched aggressively as a treatment option. Apoptosis is another process that can be used to remove actively dividing cells from system but, senescence is unique in that the process helps in retaining the functional cells in the system. 5-fluorouracil, doxorubicin, aphidicolin and cisplatin are examples of many drugs found to induce cellular senescence in tumor cells. These drugs show their effect due to the interactions with DNA (Roninson IB et al., 2001). A simple element beryllium was found to increase the levels of p53 and p21 (Lehnert et al., 2001). Cell cycle arresting properties of beryllium were first reported by Hart and Skilleter et al (Hart et al., 1982; Skilleter et al., 1983). Coates SSA et al., reported beryllium as a new premature senescence inducing drug in normal young Human Fibroblast (HFL-1) cells for the first time (Coates SSA et al., 2007).

In this study we have investigated the efficiency of beryllium as senescence inducing agent in tumor cells. Our study also shows that effects of beryllium, though very strong even at significantly lower doses, are not due DNA damage. We also found that effect of beryllium is not same on all cell lines but, depends on the sensitivity of cell line. For our investigation, we have used A172 and RKO cell lines as representatives of beryllium sensitive and resistant cell lines respectively. Beryllium is used in form of BeSO_4 , which is a highly soluble form.

MATERIALS AND METHODS

Cell Culture

Wild type A172 and RKO cells were used for experiments. The cell lines were propagated RPMI1640 with GlutaMAX supplemented with 10% fetal bovine serum and 1% PSF. The cells were cultured in 100 mm dishes at 37°C with 5% CO_2 . 0.05% trypsin EDTA was used for trypsinizing the cells when splitting.

Beryllium treatment

$\text{BeSO}_4 \cdot 4\text{H}_2\text{O}$ from Fluka was used to prepare a stock solution. Beryllium stock solution was diluted to required concentration in RPMI just before dosing.

The cells for control plates and beryllium dosed plates were taken from same cell stock and seeded to plates. After allowing the cells to attach the plates were shuffled, labeled and media changed. The control plates receive normal RPMI with no beryllium and the beryllium dosed plated receive respective concentration of beryllium RPMI.

UV and IR treatment

For ionizing radiation dosing, the Cabinet X-Ray system, model Rx-650, by Faxitron X-ray corp was used. For UV dosing a 15 W UV light source (Sankyo Denki G15T8) was used and dosage was monitored using UVX radiometer, model UVX-25.

Cell count experiment

The cells were cultured in 60 mm plates. The dishes were seeded from single cell stock and left for approximately 24 hours allowing the cells to attach. The plates are then shuffled, labeled and dosed with beryllium. 72 hours after dosing the cells were trypsinized and resuspended in RPMI with or without beryllium. Exactly same volume of cell suspension from all plates was seeded to 96-well plate. Each plate was seeded in triplicate on the 96well plate and allowed to grow for another 48 hours.

The experiment was terminated at the end of 5th day. Cell counts were obtained by measuring fluorescence using CyQUANT Cell proliferation assay kit (Invitrogen cat#C7026). Number of cells attached to plate was estimated using a standard curve generated from serial diluted cells seeded into the same plate 12hrs before termination of experiment.

Western Blot analysis

Protein extracts of cells treated with beryllium were made by in M-PER (Pierce cat#78501) with protease (Halt protease inhibitor cocktail kit, Pierce cat#78410) and phosphatase inhibitors (Sodium Fluoride 20 mM, Beta Glycerol phosphate 10 mM, Sodium ortho vanadate 0.1 mM, Paranitro phenyl phosphate 20 mM). The cell suspension was lysed by pipetting vigorously. The lysed cell suspension was centrifuged and total protein concentration in supernatant was measured by using BCA assay (Thermo scientific # 23227).

Normalized samples were loaded onto 8% gels for probing with Actin, p53 and 10% gels for probing with p21 protein levels. The gels were electrophoresed and transferred to polyvinylidene difluoride membrane (Millipore cat#IPFL20200). The following primary and secondary antibodies were used for probing.

p53: Mouse monoclonal IgG2a (Cat# SC-126 Santa cruz biotechnology, inc) and Goat anti-mouse IgG peroxidase (Cat#A9917 Sigma Aldrich)

p21: Mouse monoclonal IgG2b (Cat# SC-6246 Santa cruz biotechnology, inc) and Goat anti-mouse IgG peroxidase (Cat#A9917 Sigma Aldrich)

Actin: Goat monoclonal IgG2b (Cat# SC-1615 Santa cruz biotechnology, inc) and bovine anti-goat IgG-HRP (Cat# SC-2350 Santa cruz biotechnology, inc)

P53 ser 15: Rabbit polyclonal (Cat# 9284 Cell signaling technology) and Goat anti-rabbit IgG- HRP (Cat# 31460 Thermo Scientific)

γ H2AX: Rabbit IgG (Cat# DR1017 Calbiochem) and Goat anti-rabbit IgG- HRP (Cat# 31460 Thermo Scientific)

The blots were developed using ECL plus reagent (GE health care amersham cat# RPN 2132). Imaging was done by using Typhoon variable mode imager with excitation wavelength of 457nm and emission filter set to 520nm.

All experiments were setup in duplicates for checking reproducibility of the results.

Real time PCR

Total mRNA was extracted from control and dosed cells using Rneasy Mini Kit and QIA-shredder (Qiagen cat #74104 and 79654). Real time PCR was performed on same quantity of cDNA sample reverse transcribed from total mRNA using QuantiTect Reverse Transcription Kit (Qiagen cat# 205311) and QuantiTect SYBR green PCR kit (Qiagen cat# 204143). The primers used were Actin, GAPDH, p21 (QuantiTect Primer assays – QT00095431, QT00079247, QT00062090).

The following thermal cycling profile was used for amplification and detection - Initial Denaturation at 95°C for 15 minutes. 40 repetitions of denaturing at 94°C for 15 seconds, Annealing at 55°C for 30 seconds, extending at 72°C for 30 seconds. Final denaturation at 95°C was done for 1 minute and final extension was done at 72°C 1 minute. Melt curve analysis was done starting from 55°C for 10 seconds with increasing set point temperature after cycle 2 by 0.5°C.

Flow cytometry

The control and dosed cells were trypsinized and 5×10^5 cells were used for staining with propidium iodide. Dosed cells were treated with $10 \mu\text{M}$ BeSO_4 for 3 days. The cells were washed in cold phosphate buffered saline twice and fixed using 95% ethanol. Before staining the cells were washed in phosphate buffered saline and resuspended in 1% Triton X-100 and treated with RNase. The cells were stained with $100 \mu\text{g/mL}$ of propidium iodide.

Inductively coupled plasma mass spectrometry (ICP-MS)

A172 and RKO cells were seeded into 100 mm plates. After allowing the cells to attach the media was replaced with or without beryllium as required. 0, 10, 30 or $100 \mu\text{M}$ concentrations were used and each concentration in triplicate were setup for this experiment. After 24 hour and 48 hour time periods the media was removed and each plate washed 3 times with RPMI only (without FBS and PSF) and 3 times with phosphate buffered saline. $300 \mu\text{L}$ of M-PER buffer without protease and phosphatase inhibitors was used for each plate and cells were scrapped and transferred into 1.5 mL tubes. The samples were vortexed vigorously and centrifuged. $250 \mu\text{L}$ of supernatant was diluted by adding to 19.75 mL of millipore water and $10 \mu\text{L}$ was used for protein content estimation by BCA assay. Sample of 25 mL is filtered through 0.45 micron glass filter into a new pre-rinsed tube. A special wash control plates were also run in parallel to monitor the washing steps. Wash control plates are treated identical to the $0\mu\text{M}$ plates in setup, media changes and harvest except that, just before harvest the cells received beryllium RPMI for exactly 1 minute. Reading from these plates shows the amount of beryllium that was trapped between the cells and carried over to lysates.

Total beryllium uptake is measured by using Inductively Coupled Plasma-Mass Spectrometer. Standard curve was setup with 0, 10, 50, 100, 500, 1000 nM of beryllium in Millipore water. The readings taken were normalized to total protein content.

Senescence Associated β -Galactosidase activity assay

A172 cells were setup with doses of 0 and 10 μ M beryllium. RKO cells were dosed with 0, 10 and 100 μ M beryllium. The following HFL-1 cells were used for comparison.

HLF-1 cells at passage 16 (young) and HFL-1 passage 31 (senescent) at harvest for 6 day, HFL-1 passage 18 (young) and HFL-1 passage 32 (senescent) at harvest for 12 day assay.

Senescence Associated β -Galactosidase activity (SA β -Gal activity) was measured in protein normalized samples. The quantitative assay was performed according to method previously described (Gary and Kindell, 2005).

Fluorescent microscopy H2AX ser 139 phosphorylation

The control and test cells seeded and dosed in lab-tek chamber slides were washed with cold P.B.S, 3 times and fixed with 4% formaldehyde and permeabilized with 0.5% TritonX. The cells were then incubated in 10% Normal Goat Serum to prevent nonspecific binding. Primary anti-body treatment was given for 1 hour followed by Secondary anti-body at room temperature for 45 min in dark. ProLong gold antifade was used to seal the coverslip to slide and then the slide was taken for imaging.

Statistical Analysis

Data sets with two dosage groups at each time point were analyzed by unpaired t-test, two tailed. Data sets with more than two groups were analyzed using one-way ANOVA with a post-hoc Bonferroni's multiple comparison test ($P < 0.05$ considered significant).

RESULTS

Beryllium dosing stops tumor cells from dividing

Beryllium sulfate causes the human fibroblast cells to enter into a premature senescence in a dose dependent manner (Coates SSA et al., 2007). To investigate if

BeSO₄ has similar effect on tumor cells we have used A172 and RKO cell lines for our study. The results (Fig 4.1) show that A172 is sensitive to presence of beryllium even at 1 μM concentration where as RKO cell line is able to resist the effects even at a high concentration of 100 μM. This simple but effective cell count experiment clearly shows the difference in the effect of beryllium on the two cell lines. Death of cells in test samples is not the only reason for decreased cell counts in test samples compared to control samples. The number of cells that do not undergo cell division in test samples cumulatively resulted in lower counts at the end of 5 day time point. When observed under microscopes there were dead floating cells at higher concentration of beryllium but, at lower concentrations the test plates looked almost identical to control plates with lower confluency and lower number of mitotic cells.

Beryllium causes G1 block in tumor cells

To verify if beryllium actually causes a block in cell division we performed a flow cytometry analysis on the sensitive cell line, A172. Total of 40,000 cells from control and test samples were analyzed and counted. There is a 19% increase in cells blocked at G1 phase in 10 μM beryllium treated samples compared to controls (Fig 4.2). These results show that there is accumulation of cells in G1 phase of cell cycle when A172 cells were treated with beryllium.

Beryllium causes an increase in p53 and p21 in tumor cell lines

It is a very well established fact that increased active p53 protein in cells usually leads to G1 block of cell cycle through p21. The western blot data (Fig 4.3) shows an increase in p53 and also p21 proteins in A172 cell lines treated with 10 μM concentration for 24 and 48 hours. The increase is in correlation with the observation of G1 block in test sample made using flow cytometry data. The more resistant RKO cells on the other hand do not show any significant changes in levels of p53 or p21 proteins. RKO cells

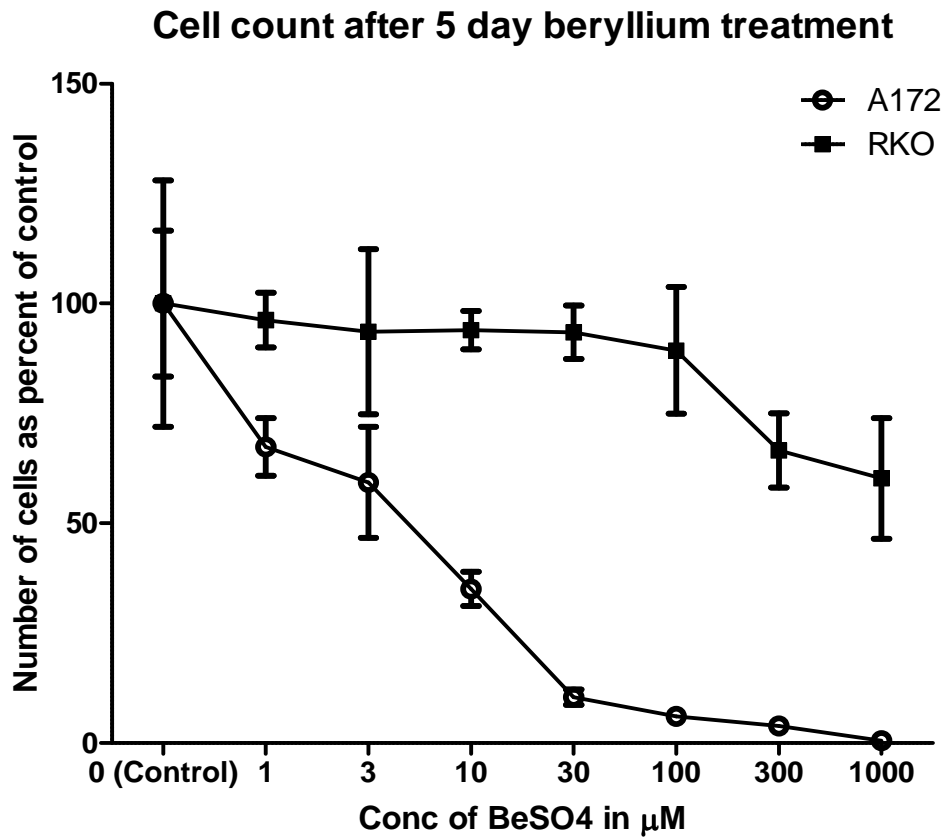
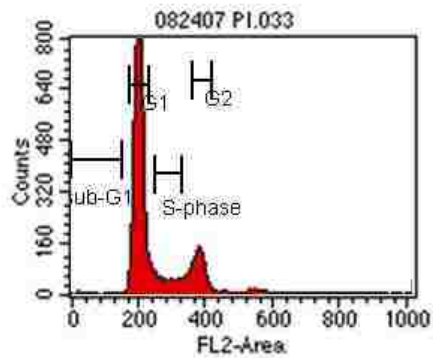
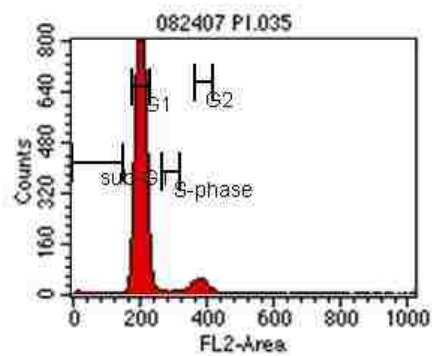


Figure 4-1 Cell count after 5 day beryllium treatment

After 5 days of treatment with beryllium RPMI, plates with A172 cells have significantly lower cells even at a very small dose of 1 μM . On the other hand RKO cells seem to tolerate a very high dose of 100 μM beryllium. The data are presented as mean +/- Standard Deviation.

A A172 Control

Marker	Events	% Total
All	40000	100
G1	27766	69.42
S-Phase	2936	7.34
G2	4236	10.59

B A172 10 μ M Be treated

Marker	Events	% Total
All	40000	100
G1	35484	88.71
S-Phase	339	0.85
G2	1447	3.62

Figure 4-2 Beryllium can induce G1-S accumulation of cells

Comparison between control (A) and cells treated with 10 μ M BeSO₄ (B) for 3 days shows that there is an accumulation of cells in G1 phase in Beryllium treated cells.

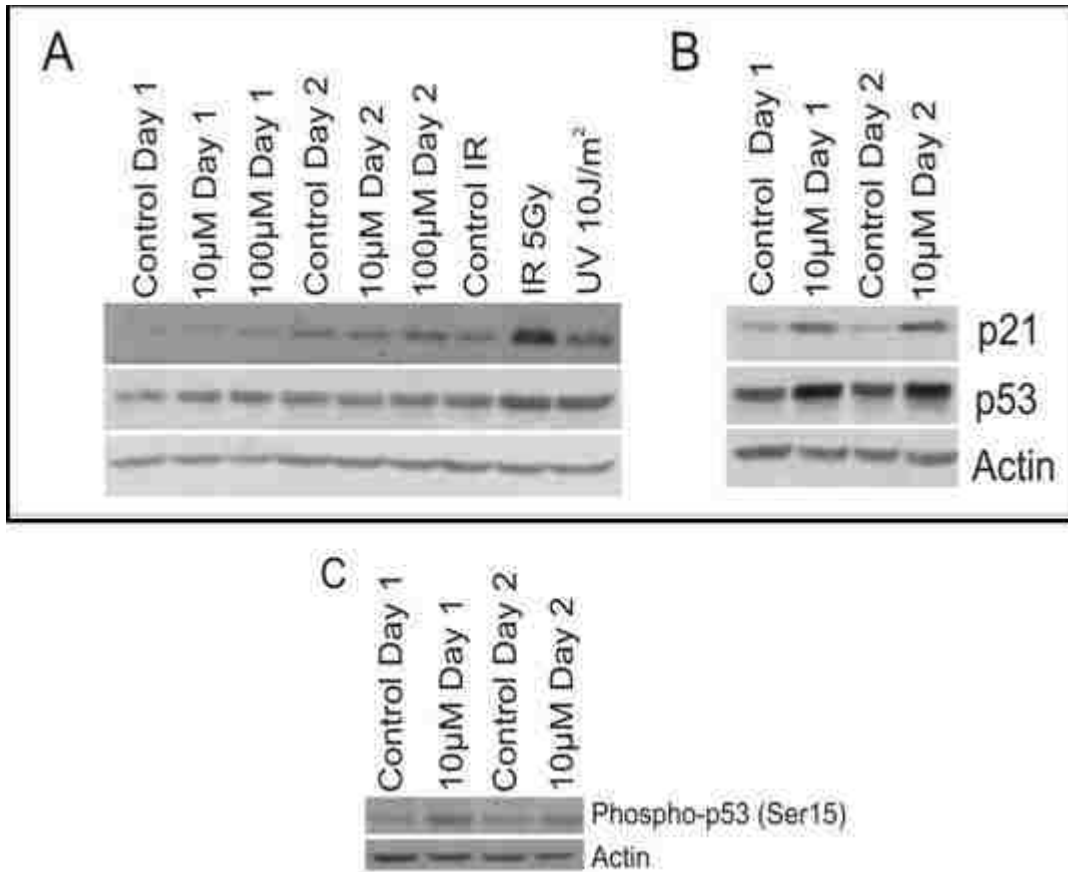


Figure 4-3 Beryllium can induce p53 and p21 in senesitive cell types

- A. RKO cells dosed with beryllium at 0 (control), 10 and 100 μM for 24 (Day 1) and 48hours (Day 2). The dosed cells show no observable change in levels of both p21 and p53 protein compared to control cells. The last two lanes show blots of lysate extracted from cells dosed with IR (X-ray at 5 gray) and UV (at 10 J/m^2) radiation. IR and UV lane extract shows increase in p53 and p21 protein proving the presence of functional p53 –p21 system in the RKO cells.
 - B. A172 cells dosed with beryllium at 0 (control) and 10 μM for 24 (Day 1) and 48hours (Day 2). Compared to control samples a very large increase in the amount of p21 and p53 protein is seen even at a very low dose of 10 μM BeSO_4 .
 - C. In a separate experiment cell lysate of A172 cells treated with 0 and 10 μM of beryllium for 24 hours and 48hours was analyzed using western blot for checking the levels of phosphorylation of p53 at ser15. The results show a clear increase in BeSO_4 treated samples
- Actin levels in the samples were used to check for protein normalization accuracy.

were treated with ionizing radiation (X-rays – 5 Gy) and UV (10 J/m²). This additional dosing shows that RKO cells had functional p53 and p21 proteins.

To measure the changes in levels of p21 Reverse transcription polymerase chain reaction (RT-PCR) is used (Fig 4.4). For the sensitive A172 cells a 135% and 225% increase compared to control is seen at 24 hrs and 48hrs respectively. The resistant RKO cells do not show increase of p21 compared to respective controls. The RKO cells have functional p21 system which is resulting in 480% and 127% increase compared to control when dosed with UV and IR respectively.

Beryllium treatment causes SA β -gal expression in tumor cells

Experiments by Coats SSA et al have shown that SA β -gal expression is increased in human fibroblast cells upon exposure to low concentrations of beryllium (Coates SSA et al., 2007). To check if a similar response is elicited in tumor cell lines, SA β -gal assay is performed on A172 and RKO cells with HFL-1 young presenescent and senescent cells as positive controls.

The increase in SA β -gal levels in A172 is comparable to response seen in HFL-1 young cells treated with same concentration of beryllium. RKO cells on the other hand have very less quantity of SA β -gal in control cells and show no change in SA β -gal levels even at a high dosing concentration of 100 μ M (Fig 4.5).

Beryllium does not cause DNA double strand breaks

Increase in concentration of p53 and subsequent increase of p21 is a typical response seen in case of DNA double strand breaks. To investigate the possibility beryllium causing DNA double strand breaks to bring about the pause in cell cycle we checked the phosphorylation of ser 139 in H2AX. H2AX is histone protein and its phosphorylation at ser 139 is used to identify cells with DNA double strand breaks. DNA damaging agents Etoposide, Camptothecin and UV dosing were used to generate

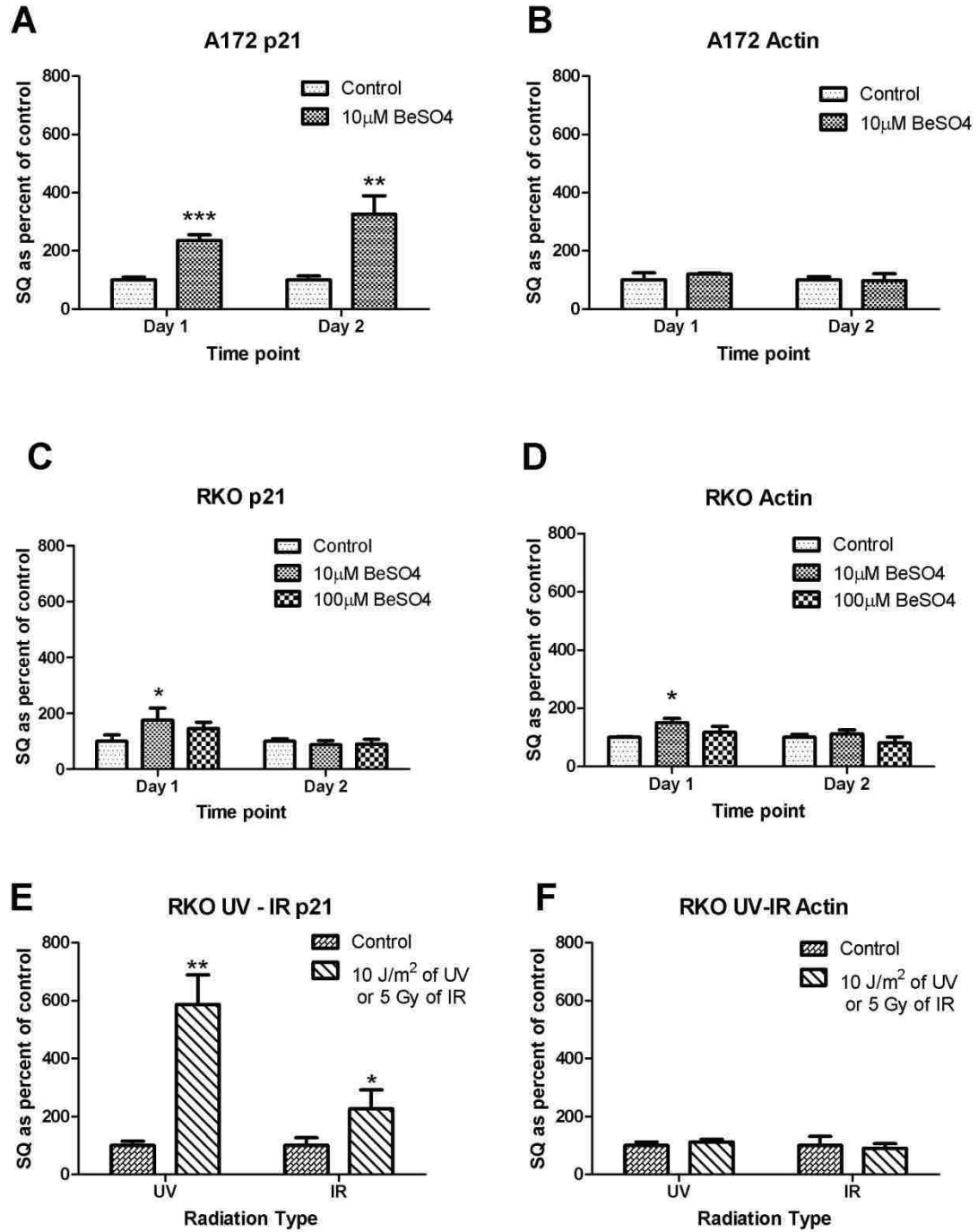


Figure 4-4 Beryllium causes changes in p21 gene expression

A. A172 cells were treated with 0(control) and 10 μ M beryllium and GAPDH and p21 levels of mRNA were measured. From the plot it is obvious that there is significant increase in mRNA levels of p21 in beryllium dosed cells.

C. RKO cells were treated with 0(control), 10 μ M and 100 μ M. The mRNA levels measured using Real time PCR shows that, there is no significant increase in p21 even at 100 μ M dose of beryllium.

E. To verify if RKO cells have has functional p21 genes RKO cells were exposed to UV, IR and mRNA levels measured.

B,D,F Actin is a house keeping gene used and GAPDH mRNA levels were used to normalize the data.

The data are presented as mean +/- Standard Deviation. Statistically significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

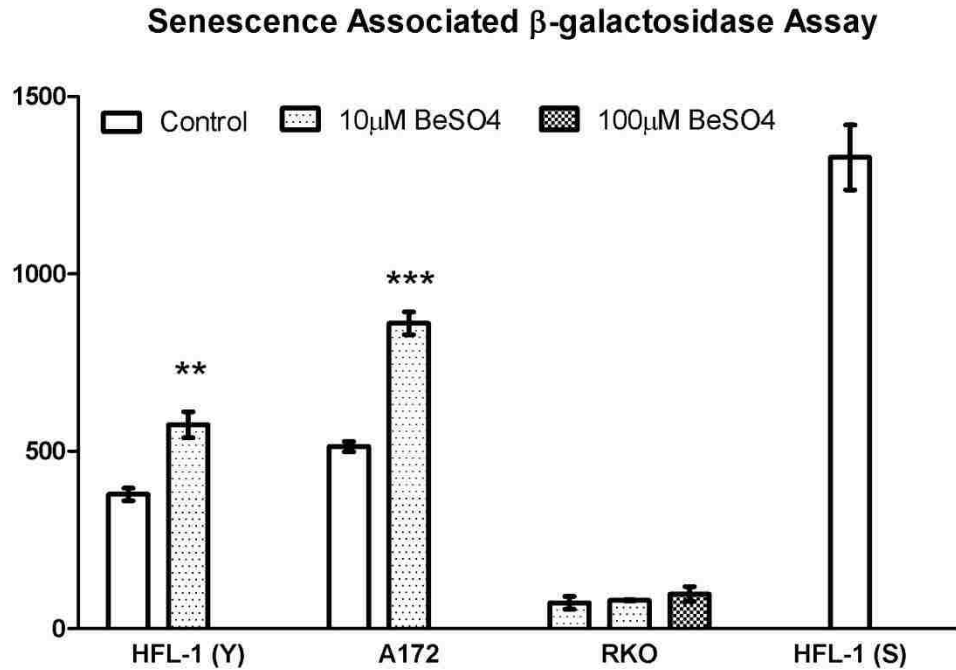


Figure 4-5 SA β -gal is induced in sensitive cancer cell types by beryllium

Fluorescence readings were taken at the end 12 day treatments on cell lysates at 0,30,60,90,120 minute time points. The data presented here is from reading taken at the end of 120 minutes. The signal was strongest at end of 120 minutes and the signal strength at other time points was relatively weak but proportional to the data shown here. HFL-1 senescent cells were generated by propagating the young cells till they reached passage 35 by the time of harvest. HFL-1 used as positive control for the assay. A172 Young cells were at passage 18 by the time of harvest. The young HFL cells were treated with 10 μ M and cells were treated with 10 μ M beryllium and RKO cells were given a low dose of 10 μ M and high dose of 100 μ M beryllium.

The levels of SA β -gal levels were measured by using 4-methylumbelliferyl- β -d-galactopyranoside (MUG). The final values of fluorescence are normalized to the total protein content of the respective samples. The data are presented as mean \pm Standard Deviation. Statistically significant differences between control and treatment groups are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

positive control samples for western blot and etoposide only for immunofluorescence experiments.

Both 24 and 48 hours treatment even at a very high concentration of 300 μ M beryllium failed to show any increase of H2AX ser 139 phosphorylation. The positive controls, Etoposide, camptothecin at 10 μ M each for 24 hours and UV dosed samples, on the other hand have shown significant increase of H2AX ser 139 phosphorylation (Fig 4.6).

Immunofluorescence microscopy is usually used to check for Ser 139 phosphorylation in H2AX. Our experiments with 10 μ M and 300 μ M beryllium for 24 hours have resulted in images looking similar to control samples while bright foci can be seen in positive control i.e., 10 μ M Etoposide samples.

Both Western blotting and Immunofluorescence microscopy results show that BeSO₄ even a very high concentration of 300 μ M may not be inducing any double strand breaks.

All the observations made in the above experiments show that A172 is affected by beryllium more than RKO. ICP-MS was used to measure the intracellular concentration of beryllium in each cell line (Fig 4.7). This was done to find out if there is any difference in uptake of beryllium by the cell lines that is resulting in the difference in sensitivities. The final values of beryllium concentration inside the cells were normalized to the total protein content of the cells used for experiment. At a lower concentration of 10 μ M A172 cells appear to be taking up more beryllium compared to RKO cells but, at higher concentration of 100 μ M both cell lines are taking up approximately equal amount of the drug (Fig 4.7). The various effects of beryllium observed at 100 μ M in RKO cells are insignificant compared to the response shown by A172 at 10 μ M concentration (Fig 4.1, 4.3, 4.4, 4.5). These two observations, almost same amount of beryllium uptake but difference in response of the two cell lines suggest that beryllium may be influencing a

pathway that is present is A172 but not in RKO. This suggests that the difference in uptake of beryllium is not a cause for the observed difference in the effects between the two cell lines

DISCUSSION

Constant long term exposure to beryllium will show its effects as chronic beryllium disease which in some cases may be lethal. Beryllium is carcinogenic at high concentrations but has the ability to induce premature senescence at lower doses (Coates SSA et al., 2007).

Our initial experiments with tumor cells and beryllium show that it is able to stop tumor cells from proliferating (Figure 4.1). Beryllium shows difference in its effects depending on the type of cell line. The decrease in cell proliferation is more in sensitive cell line A172 than the resistant line RKO. Only the cells that are attached to the dish are used to generate the cell count of viable cells. The decrease in cell count at lower doses is not due the cell death as it appears from the graph (additional data not shown). The lower cell counts in beryllium treated samples can be explained by arrest of cell division at G1 phase (Figure 4.2). The major contributor for decrease in counts in the cell count experiment is the decrease in cells that undergo division.

One significant event that occurs within 24 hours after treating tumor cells is increase in p53 and p21 proteins (Figure 4.3). The change is more pronounced in A172 cells compared to RKO. p53 protein shows its effect on cell cycle by activating transcription of p21, a very important protein downstream of the p53 controlled senescent or apoptosis pathways.

The amount of available active p53 inside the cell is increased by phosphorylation of residues at different positions with help of various kinases. These changes prevent degradation of p53 resulting in its accumulation. Status of Ser 15 phosphorylation is verified to estimate stabilization of p53 (Shieh SY et al., 1997). We

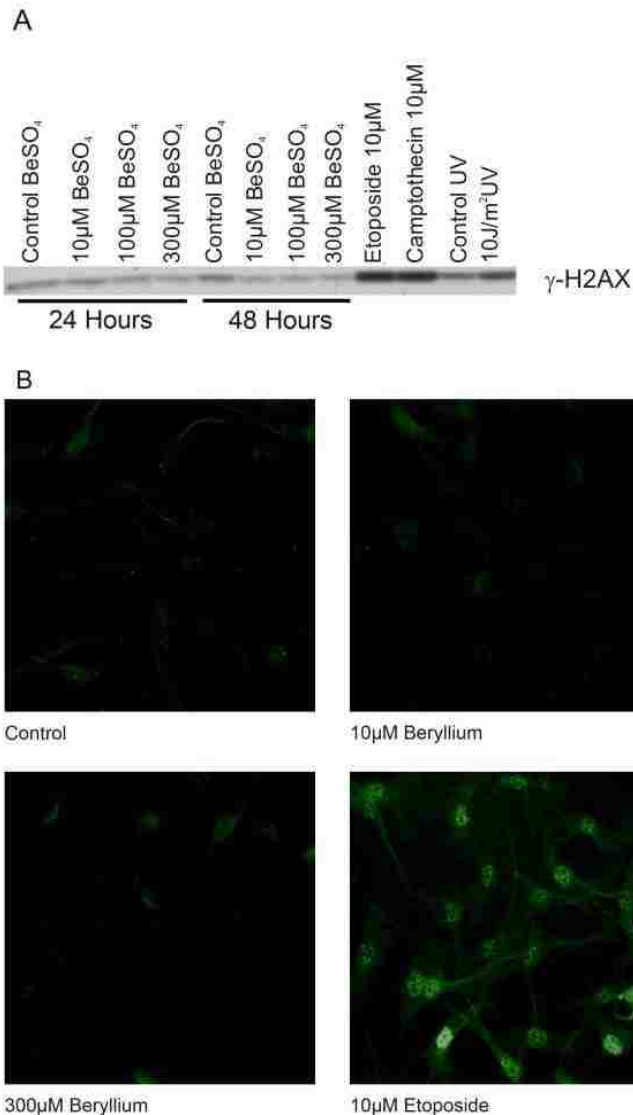


Figure 4-6 Beryllium does not cause DNA damage

- A. A172 cells were treated for 24 and 48 hour period with the above shown doses of beryllium. Cells treated with 10 µM concentration of Etoposide and Camptothecin for 24 hours along with UV dosed cells were used as positive controls. The lanes with beryllium treated samples show no increase in H2AX ser 139 phosphorylation compared to negative control lanes. The positive control samples treated with Etoposide, Camptothecin and UV light show enormous increase in H2AX Ser 139 phosphorylation.
- B. A172 cells treated with 0µM used as control and 10 µM or 300µM beryllium used as test and 10 µM Etoposide as positive control were analyzed using immunofluorescence microscopy. The cells were fixed, permeabilized and treated with primary antibody against gamma H2AX and a FITC bound secondary antibody. Double strand DNA breaks trigger phosphorylation of H2AX at ser 139 position and will be seen as bright foci, as in case of Etoposide 10 µM treated cells.

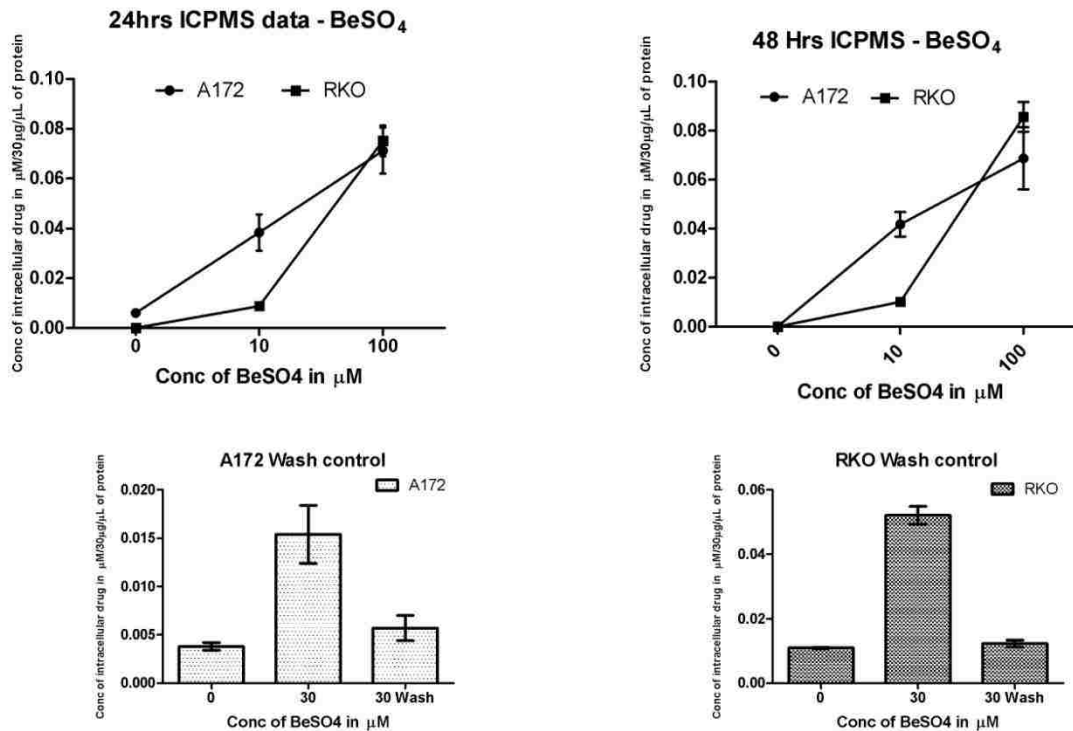


Figure 4-7 Differential uptake of beryllium is not a cause for difference in A172 and RKO sensitivities

At 10 μM beryllium concentration, it appears that A172 cell line is taking up higher beryllium compared to RKO cell line. But the 100 μM data shows clearly that difference in uptake of the beryllium is not significant between the two cell lines.

The wash control data demonstrates the efficiency of wash procedure in removing Be^{2+} from the exterior surface of cells. The cells used for 30 wash control are cultured in 0 μM but exposed to BeSO_4 for 1 minute then washed according to protocol and prepared for ICP-MS analysis.

The data are presented as mean \pm Standard Deviation.

found that beryllium treatment causes phosphorylation of p53 at ser15 (Figure 4.3C). p21 levels are increased by transcribing p21 mRNA which is regulated by stabilized p53. The increase in p21 protein levels corresponds to the increase in p21 mRNA levels (Fig 4.4). The amount of p21 mRNA is 135% more at 24 hours and 225% more at 48 hours compared to control. The RKO cells show no change even a 100 μ M, 10 times higher dose than the concentration required bringing about a response in A172. RKO cells have functional p53 - p21 system which is evident from the response to UV and IR dosing verified by western blotting and RT-PCR experimental data. These changes are in agreement to a well documented observation that initial increase in p21 inhibits progression of cell cycle in the cells (Di Leonardo, A. et al, 1994).

SA- β -gal is used as an important biomarker for senescence. It is found to be increased only in senescent cells and not in actively dividing or terminally differentiated cells (Dimri GP, 1995). Recent studies show that drug induced increase in levels of SA- β -gal can be used as marker for estimating the senescent like cell cycle arrest in cancer cells. The increased level of senescence-associated beta-galactosidase seen in these senescent cells is due to the accumulation lysosomal β -galactosidase (Lee BY et al., 2006). Increased lysosomal activity is observed in the cells that are senescent stage (Gerland et al., 2003). Our hypothesis that beryllium induces senescent-like state in cancer cells is corroborated by the recent finding that suggests intracellular concentration of beryllium may not be able to induce apoptosis (Ding J et al., 2009). We have found that long term treatment of A172 cells with beryllium induces expression of SA- β -gal (Fig 4.5). The level of SA- β -gal expressed is comparable to that seen in young HFL-1 cells. RKO on the other hand did not show any change in SA- β -gal levels. No change of SA- β -gal level in beryllium treated RKO samples is consistent with the actively dividing cells seen in that cell line.

The drugs that are used in therapy for cancer for their ability to induce senescence usually cause DNA damage. The DNA damage subsequently results in activation of p53 based pathway to induce senescence. DNA damage can be monitored by checking the phosphorylation of ser 139 residue on histone H2AX (Rogakou EP et al., 1998). We have used H2AX Ser 139 phosphorylation as marker for DNA strand break to verify if beryllium was causing DNA damaging in the sensitive cell line i.e., A172. Cell cycle arrest-inducing low dose of BeSO₄ at 10 μM and a very high dose of 100 μM and 300 μM were used. Results from both western blotting as well as immunofluorescence suggest that beryllium does not cause any DNA damage.

The difference in magnitude of the effect of beryllium observed in A172 and RKO is not due to difference in uptake of beryllium as evident from ICP-MS study. It is possible that the difference is due to the variable genetic sensitivities of the cell lines. In the present study we were able to confirm that beryllium is able to induce senescence in sensitive cancer cell lines. The mechanism by which Be²⁺ is doing this is not yet clear but the results indicate that the mechanism involves no DNA damage that is seen regularly with other senescence inducing drugs. The unique ability of beryllium to activate senescence signaling pathway through p53 without inflicting DNA damage makes it novel tool to investigate the mechanism of senescence induction in greater detail.

CHAPTER 5 CELL CYCLE INHIBITING EFFECTS OF BERYLLIUM ON CANCER CELLS ARE P53 DEPENDENT

INTRODUCTION

Beryllium, when administered to cells in the form of beryllium salts was found to inhibit the growth in human fetal lung fibroblast and rat hepatic cell lines. Cell cycle arresting properties of beryllium were first reported by Hart and Skilleter et al (Hart et al., 1982; Skilleter et al., 1983). Beryllium in the form of BeSO₄ was reported as a new premature senescence inducing agent in normal young Human Fibroblast (HFL-1) cells. Further, it was demonstrated that BeSO₄ was influencing the cell cycle progression while causing an increase of cell cycle inhibitor protein p21 and a decrease in DNA synthesis, resulting in an induced premature senescence. BeSO₄ was found to increase the levels of p53 and p21 and inhibit growth in normal human lung fibroblast (Lehnert et al., 2001). It was also discovered that p53 protein binds to the promoter region of p21 thus regulating its transcription in the presence of BeSO₄ (Coates et al., 2007). In an earlier study we have investigated the ability of BeSO₄ to induce senescence like state in susceptible cancer cell lines. BeSO₄ was found to stabilize p53 and induce expression of p21 followed by induction of senescence like state in some cancer cell lines with wild type p53 without considerable effect on other cell types (Gorjala and Gary, 2010). This observation suggested that presence of wild type p53 alone was not sufficient to elicit a response to beryllium salt treatment.

p53 protein plays a key role in the intracellular signaling network in sensing the DNA damage and regulating the cell cycle (Di Leonardo et al., 1994). p53 through p21 mediates senescence in normal cells and is also responsible for senescence in cancer cells when exposed to drugs (Chang et al., 1999). p53 can be activated by various stress stimuli, DNA damage, short telomeres or activated oncogenes. The activated p53 can induce expression of p21 which in turn can mediate cell cycle arresting actions (EI-

Deiry et al., 1993; Xiong et al., 1993). Studies have established that p21 can be expressed in both p53-dependent as well as p53-independent manner (Macleod et al., 1995). Further it has been found that p21 can be up-regulated in p53 independent manner but p53 is required for p21 induction in situations where DNA damage is caused by radiation (El-Deiry et al., 1994; Michieli et al., 1994).

Beryllium has been shown to introduce G1-S phase cell cycle block (Skilleter et al., 1983; Lehnert et al., 2001). Cyclin-dependent kinases (CDKs) are a family of protein kinases that are periodically activated during cell cycle due to binding of proteins called cyclins. DNA damage or other genotoxic stress induces p21Waf1/Cip1 which binds to cyclin E-cdk2 complex. Binding of p21 to cyclin E-cdk2 complex results in competitive inhibition blocking S-phase entry to cells due to hypophosphorylation of pRb (Morgan, 1996; Sherr and Roberts, 1995). Preliminary results from microarray data suggest that beryllium causes a decrease in cyclin E2 mRNA levels (unpublished data).

A172 cells, a glioblastoma cell line, which are used for this study express wild type p53 sequence and have functional p53 protein (Ishii et al., 2006; Jia et al., 1997; Mirzayans et al., 2005). Beryllium is able to increase p53 and p21 in A172 cancer cells without DNA damage (Gorjala and Gary, 2010). In the present study we have investigated whether p21 induction and cell cycle arrest produced by beryllium is p53 dependent. We have used three different “knock-down” methods, siRNA, shRNA and expression of E6 protein, to remove p53 protein or mRNA from cells. Short interfering RNA (siRNA) is an approximately 20 nucleotide long synthetic double stranded RNA molecule that can post-transcriptionally silence a gene. siRNA method has ready to use reagents and is the quickest method to knock down a specific gene. The protocol can be optimized to minimize off-target nonspecific effects but the knock down is transient and potent for short term experiments. On the other hand short hairpin RNA (shRNA) has a sequence inserted into viral vector plasmids. Expression of shRNA in target cells causes

silencing of target gene expression. shRNA provides an alternative to avoid working with RNA and can induce a stable expression. The disadvantages of using shRNA include the need for time consuming cloning and the need for verification of insert. The third method used is E6 protein expressed by HPV that binds to the p53 protein and promotes its degradation in ATP dependent manner through ubiquitin-protease system (Scheffner et al., 1990). Each of the knock down methods removes either the mRNA transcript or functional protein from the cells. The result of each knock down method individually often is not completely effective in removing the gene product. On the other hand the “knock-out” methods involve deletion of the target gene. By using three knock down methods in the same study we were able to complement the results from one method with the other and get a complete picture.

MATERIALS AND METHODS

Cell lines and reagents

A172 (human glioblastoma) cells were from ATCC (Manassas, VA). Cells were grown in RPMI 1640 with Gluta-MAX and 25 mM HEPES (Invitrogen-Gibco) supplemented with 10% FBS (HyClone) and 1% Antibiotic–Antimycotic (Invitrogen-Gibco) at 37°C in a 5% CO₂ atmosphere. BeSO₄·4H₂O used for dosing cells was from Fluka (a division of Sigma-Aldrich).

A172-E6 cells are A172 cells transfected with pCMV-E6 expressing the human papillomavirus 16 E6 gene. A172-neo cells are A172 cells transfected with a similar plasmid without E6 (pCMV-neo) to serve as transfection control. Both A172-E6 and A172-neo are established and characterized as described earlier (Xu et al., 2005) and were kindly provided by Dr. J. Gregory Cairncross (University of Calgary, Calgary, Alberta, Canada).

siRNA based p53 mRNA knockdown was done by using Validated Stealth RNAi DuoPak (Cat # 45-1492). Additional reagents used in the procedure were Lipofectamine

2000 (Cat # 11668-027) and OptiMEM reduced serum RPMI (Cat # 11058-02). Each of the Stealth RNAi molecules from Stealth RNAi DuoPak were used at a final concentration of 20 nM and Lipofectamine 2000 was used at 0.01 mg/mL. The reagents were diluted from stocks in OptiMEM. Each 100 mm plate received 2.5 mL of mixture for 12 hours. The cells were then trypsinized and reseeded at required confluency and dosed with BeSO₄.

shRNA based p53 knock down was achieved by transducing A172 cells with p53 shRNA (h) Lentiviral Particles (Cat#sc-29435-V) and transduction control cells were generated by using Control shRNA Lentiviral Particles (Cat# sc-108080) on A172 cells. Successfully transfected cells were selected by growing them in RPMI with puromycin followed by isolation of single cell clones.

Cell count experiment

50,000 cells were seeded to 60mm plate for each dose. The cells were given change of media with BeSO₄.4H₂O at required concentrations. Cells were trypsinized by adding 0.5mL of trypsin EDTA followed by incubation at 37°C and 5% CO₂ for 3 mins. The cells were resuspended in 2.5mL of RPMI. 100µL of cell suspension was used for cell counts. Remaining cell suspension was used to seed cells in to fresh 60mm plate at predetermined split volume to maintain subconfluency. Cell counts were taken using a Beckman Coulter Z1 counter.

mRNA quantification by RT-PCR

RNA from treated cells was isolated using RNeasy kit, cDNA was synthesized and Real Time-PCR was performed using QuantiTect SYBR Green PCR reagents from Qiagen. Primer pairs were, also from Qiagen - QuantiTect Primer Assay, QT00095431, QT00079247, QT00062090, QT000060235, QT00041986, QT01012284 for human beta-actin, human GAPDH, human p21(CDKN1A), human p53, human cyclin E1 and cyclin E2 respectively were used. Thermal cycling and data collection was performed on

a Bio-Rad iCycler. iCycler software was used to determine PCR efficiency for each gene-specific primer set and to calculate the Starting Quantity (SQ) of gene-specific mRNA in each sample. Data was analyzed according to the method described earlier (Gorjala and Gary, 2010).

Western blotting

Cells ready for harvest were washed after RPMI was removed. Cell lysis was performed in plates by addition of 300µL of M-PER (Mammalian Protein Extraction Reagent, Pierce) supplemented with protease and phosphatase inhibitors. Total protein concentration was determined by using a bicinchoninic acid (BCA) assay (Pierce) and equal amount of total protein sample per well was resolved on precast Bis-Tris Midi Gel and transferred to PVDF membrane. Immunoblotting was conducted using anti-p53 mouse monoclonal (sc-126, clone DO-1), anti-p21 mouse monoclonal (sc-6246, clone F-5), anti-actin goat polyclonal (sc-1615) antibodies from Santa Cruz Biotechnology, Inc, CA. Appropriate HRP-conjugated secondary antibodies were used and blots were developed with ECL Plus (GE Healthcare) imaged on a Typhoon Variable Mode Imager set to scan in fluorescence mode with a 457 nm laser and emission at 520 nm. Some of the blots were developed using Immun-Star™ WesternC™ Chemiluminescence Kit and images were taken using ChemiDoc XRS+ from Bio-Rad.

Cell cycle analysis by flow cytometry

After cell culture, the cells were fixed with 70% ethanol, treated with RNase, and stained with 50µg/ml propidium iodide (PI). Cellular DNA content was determined from PI fluorescence intensity using a Becton–Dickinson FACSCalibur flow cytometer and Flowjo data analysis software for cell cycle phase determination.

DNA synthesis and total DNA content

Cellular DNA synthesis was measured from the incorporation of 5-bromo-20-deoxyuridine (BrdU). Cells were grown in the presence or absence of BeSO₄ for 72 h.

Cells were allowed to grow for 61 h in 100mm plates. The cells were trypsinized, counted and re-seeded into 96-well plates at constant number into each well. Two identical 96-well plates were setup. The cells were allowed to and at the end of 72 hours BrdU label was added. Label incorporation was allowed for 3.5 hours and 96-well plate were processed and fluorescence was measured using a plate reader set to 340 nm excitation, 465 nm emission.

RESULTS

Three methods to knock down p53 mRNA or protein

To generate A172 cell lines lacking p53 activity three different strategies were employed.

Two different validated stealth siRNA sequences targeted to TP53 mRNA (accession number NM_000546) coding region between 252-1433 were used to induce knock down of p53 transcript. The resulting cells are referred to as KO1, KO2 and siRNA control treatment cells as KO control. We observed on western blot that the transfection target p53 protein is almost completely removed from the cells. The effect of siRNA though transient was effective in keeping the levels of p53 down even at the end of 48hours (Fig 5.1a).

As second approach we established sub-clones of A172 cells by infecting them with Lentiviral particles containing expression constructs encoding target-specific 19-25nt shRNA to knockdown gene expression of p53. The sub-clones of A172 constitutively express shRNA specific for p53. After transduction, cells expressing the constructs were selected under puromycin pressure. Single cell clones were isolated and propagated. Western blotting done on cell lysates of the clone used for this study (Fig 5.1b) show significantly reduced p53 protein compared to normal A172 cells. The established p53 knocked down clones are called as LVp53KO and transfection control clones as LVp53KOcontrol.

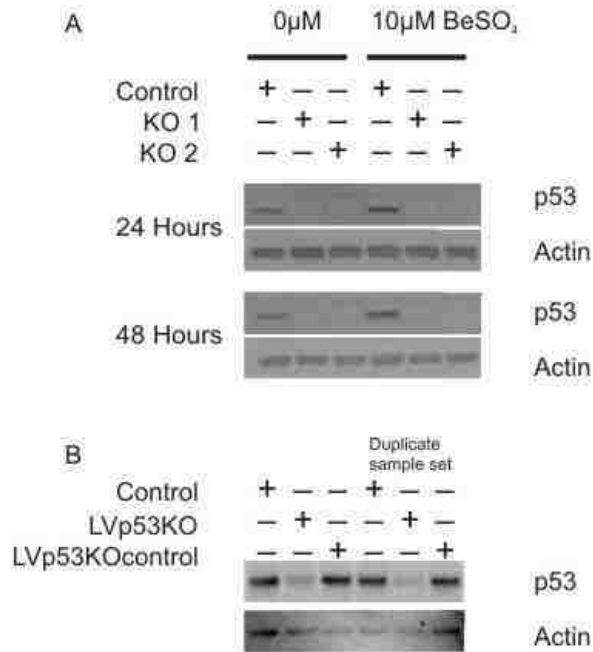


Figure 5-1 Downregulation of p53 protein siRNA and shRNA

- Two different validated stealth RNAi sequences (KO1 and KO2) were used to knock down p53 mRNA in A172 cell lines. Western blotting was used to evaluate levels of p53 and Actin protein in A172 normal, A172 KO1 and KO2 treated with 0 and 10 μM BeSO₄ for 24 and 48 hours.
- Reduced expression of p53 protein in A172 cells, LVp53KO. The p53 gene expression is knocked down by using p53 shRNA (h) Lentiviral Particles. Duplicate sample set is shown here.

Both the above methods work on mRNA to remove p53 transcripts from the cells. A third strategy used was expression of E6 protein from human papillomavirus 16. The E6 protein expressed by HPV binds to the p53 protein and promotes its degradation in ATP dependent manner through ubiquitin-protease system (Scheffner et al., 1990; Vogelstein and Kinzler, 1992).

Functional p53 makes A172 cells sensitive to growth inhibition by beryllium

Beryllium was able to inhibit cell proliferation in the presence of functional p53 protein. A172 cells that were expressing p53 i.e., A172, A172 KO control (Fig 5.2a) and A172, A172-neo in (Fig 5.2b) were very sensitive to 10 μ M beryllium. Whereas cells with p53 knocked down showed tolerance to beryllium relative to cells with functional p53.

Cumulative cell number of the three cell types A172 (functional p53), A172 LVp53KOcontrol, A172 LVp53KO was almost same under 0 μ M beryllium (Fig 5.2c). In the presence of 10 μ M beryllium the LVp53KO cells showed sustained increase in cell number over a period of 12 days but the cell types with functional p53 showed cytostatic response for up to 9 days followed by a decrease in cell number (Fig 5.2c).

Beryllium induced accumulation of cells at G1-S phase and decrease in DNA synthesis is p53 dependent

BeSO₄ causes G₁-S phase block in A172 cells (Gorjala and Gary, 2010). Cell cycle analysis by flow cytometry confirmed this result in A172 and A172-neo cell lines. The percentage of cells in S phase at 0 μ M dose in A172 dropped from 15.1% +/- 1.1 to 5.3 +/- 1.1 in 10 μ M treated samples. Similarly the percentage of cells in S phase in A172-neo cell type changed from 9.4 +/- 1.1 to 1.2 +/- 0.2 between 0 and 10 μ M doses. However the percentage of cells entering S-phase changed from 13.3% +/- 0.7 to 10.8 +/- 0.4 with 10 μ M beryllium treatment in A172-E6 cells which have low levels of functional p53 compared to A172 and A172-neo (Fig 5.3a).

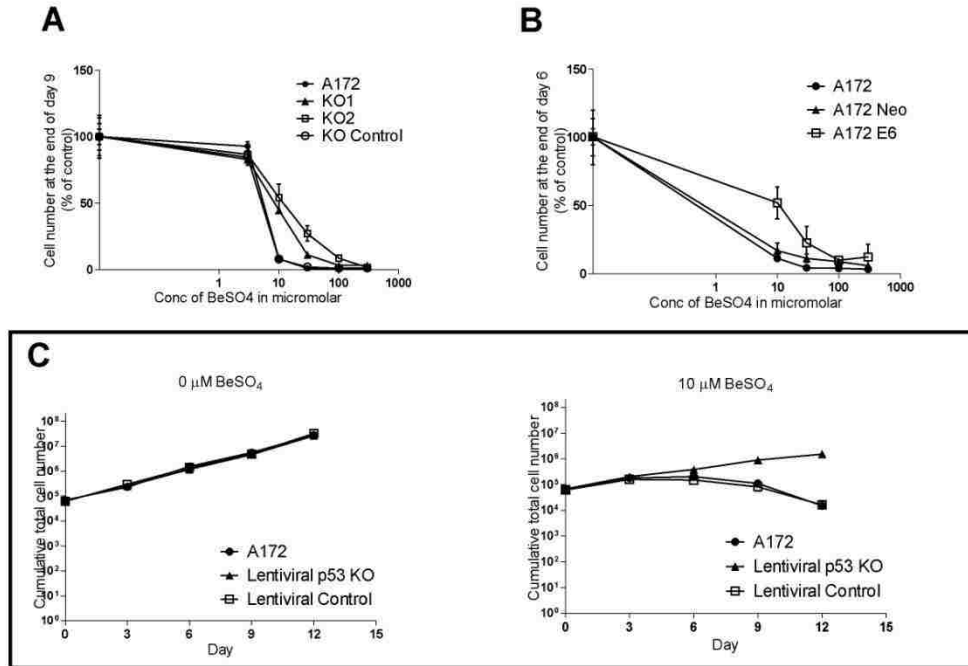


Figure 5-2 Presence of functional p53 sensitizes cells to growth inhibition by beryllium

- A172 cells and p53 siRNA treated A172 cells (KO1, KO2, KO Control) were cultured RPMI with 0, 3, 10, 30, 100 μM BeSO_4 and counted at the end of 9 days and shown here as percent of untreated cells (mean \pm SD) of respective cell types.
- A172 cells, E6 expressing A172 cells and A172 Neo cells were cultured in RPMI with 0, 10, 30, 100 μM BeSO_4 and counted at the end of 6 days. Counts are expressed as percent on untreated cell number (mean \pm SD) of respective cell types.
- A172 cells with functional p53 (A172 and lentiviral control) or with p53 knocked down (Lentiviral p53 KO) were cultured in 0 μM BeSO_4 RPMI (left) and 10 μM BeSO_4 (right) for 12 days. Cumulative cell number (mean \pm SD) was determined every 3 days and cells were re-seeded to fresh plates to maintain sub confluency.

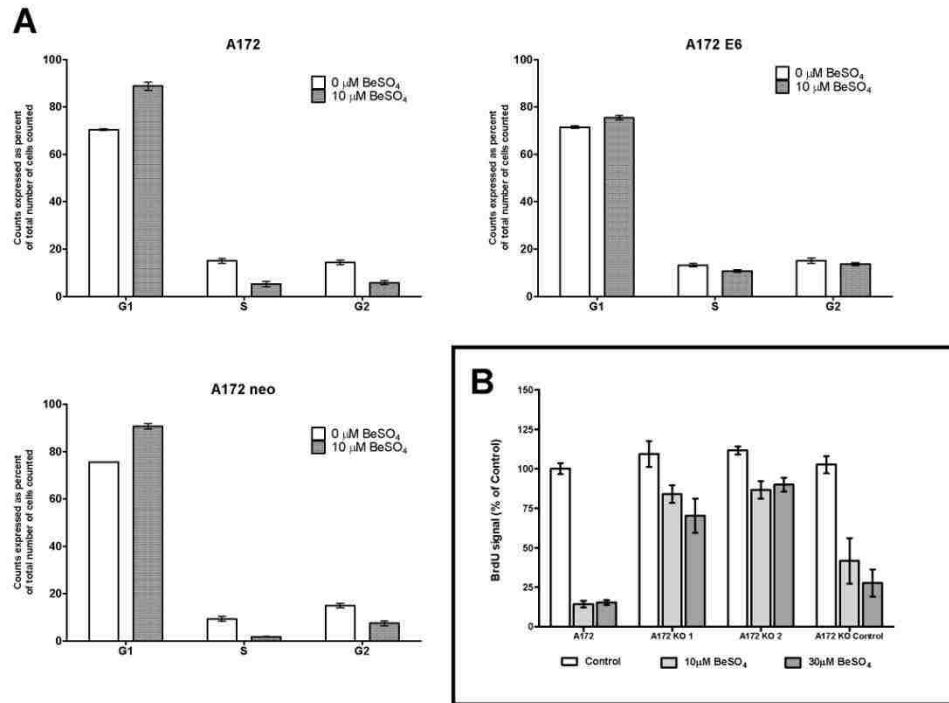


Figure 5-3 Beryllium induced accumulation of cells in G1-S phase and DNA synthesis block requires functional p53 protein

The percentage of cells with p53 protein knocked down entering S-phase in the presence of BeSO_4 is more compared to cells with functional p53. This observation also correlates with higher DNA synthesis activity in p53 knockdown cells compared to normal cells.

- A172 normal, A172-E6 and A172-neo cells were cultured in RPMI +/- 10 μM BeSO_4 for 72hours. Propidium Iodide (PI) stain was used to measure DNA content using flowcytometry. Data is expressed as percentage of mean \pm SD of total cells number of counted from three replicate samples.
- A172 cells were treated with siRNA to knock down p53 mRNA (KO1, KO2 and KO control serves as siRNA treatment control). A172 cells and siRNA treated A172 were cultured in RPMI with 0, 10, 30 μM BeSO_4 for 72hours. Rate of DNA synthesis was analyzed by using BrdU incorporation assay. Values are expressed as mean \pm SD of percent of A172 0 μM BrdU fluorescence signal measured

DNA synthesis occurs during S phase of cell cycle. Beryllium can induce and maintain a reversible decrease of DNA synthesis (Gorjala and Gary, 2010). A172, KO1, KO2 and KO control cells were treated with 10 μ M beryllium for 72 hours. The rate of DNA synthesis was measured using incorporation of BrdU, a thymidine analog (Fig 5.3b). In normal A172 cells, the rate of DNA synthesis in 10 μ M and 30 μ M BeSO₄ cells decreased to 15% \pm 2 and 15% \pm 2 of control cells. At 10 μ M dose of beryllium A172 cells with p53 knocked down i.e., KO1 and KO2, showed a small decrease to 84% \pm 5 and 86% \pm 5 relative to A172 cells at 0 μ M.

Beryllium induced p21 increase is dependent on p53 protein

p21^{waf/cip} is an important cyclin-dependent kinase inhibitor that regulates the progress of cell cycle. p21 is known to function in both p53 dependent as well as independent manner. To evaluate significance of p53 in the cell cycle inhibition response of A172 cells to beryllium treatment, we have analyzed the mRNA and protein levels of p21. p21 is a product of *CDKN1A* gene that is involved in cell cycle inhibition and a target for p53 transcription factor.

A172, KO1, KO2 and KO control cells were cultured in RPMI with 0 and 10 μ M BeSO₄. mRNA levels for p53 and p21 were measured using quantitative RT PCR. The siRNA knock down of p53 gene expression was effective and is evident from p53 mRNA decrease to 46.0% and 33.6% of control in KO1 and KO2 respectively but no change seen in KO control cells (Fig 5.4). The p21 mRNA decreased to 55.4% and 34.9% of control in KO1 and KO2 cells treated with 10 μ M BeSO₄ but did not change in control and control KO cells. The A172 cells when treated with 10 μ M beryllium showed 4 times increase in p21 mRNA. In A172 KO1 and KO2 the p21 levels increased modestly to 122.99% and 91.84% of A172 control at 0 μ M treatments. Similar response was also observed in A172-E6 cells showing a small change compared to control cells in which p21 mRNA increased significantly (Fig 5.5). Although the expression of p53 mRNA in

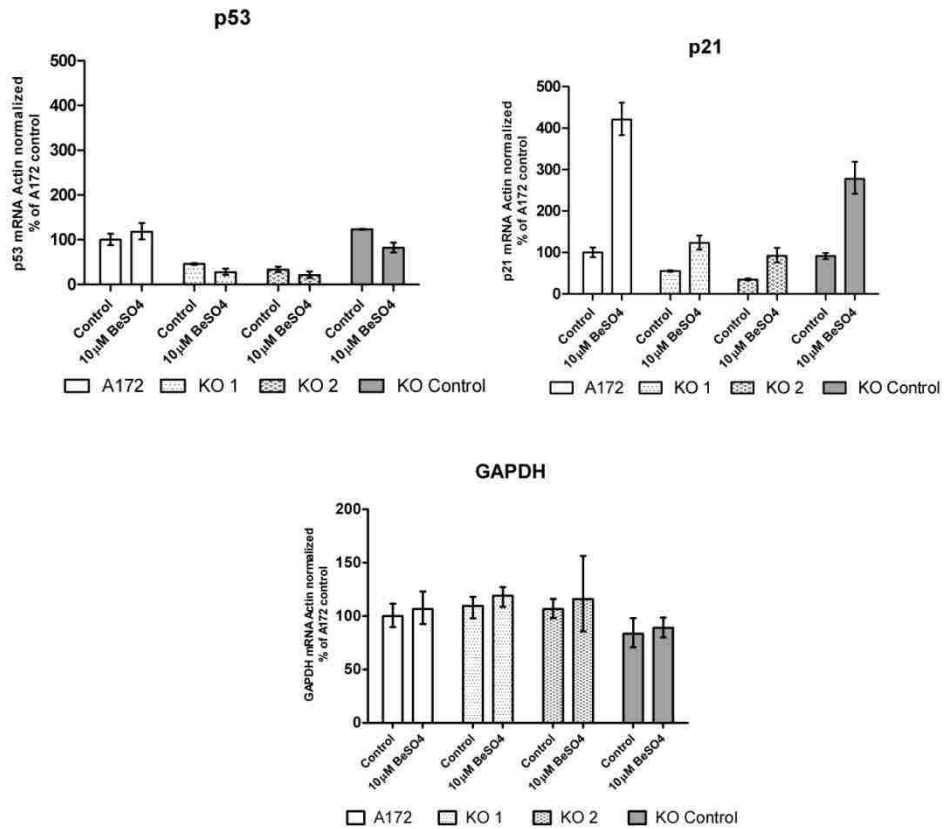


Figure 5-4 BeSO₄ up-regulation of p21 in p53 dependent manner

A172 cells were treated with siRNA to induce p53 mRNA knock down. The cells are then cultured in the presence of 0 or 10 μM BeSO₄ for 48 hours. mRNA levels of p53, p21, Actin and GAPDH in each sample were quantified using RT-PCR. Housekeeping genes Actin and GAPDH are expected to generate relatively constant amount of mRNA in control and treatment samples. mRNA quantities of all genes quantified were normalized relative to Actin mRNA and expressed as mean +/- SD of percent of A172 control values.

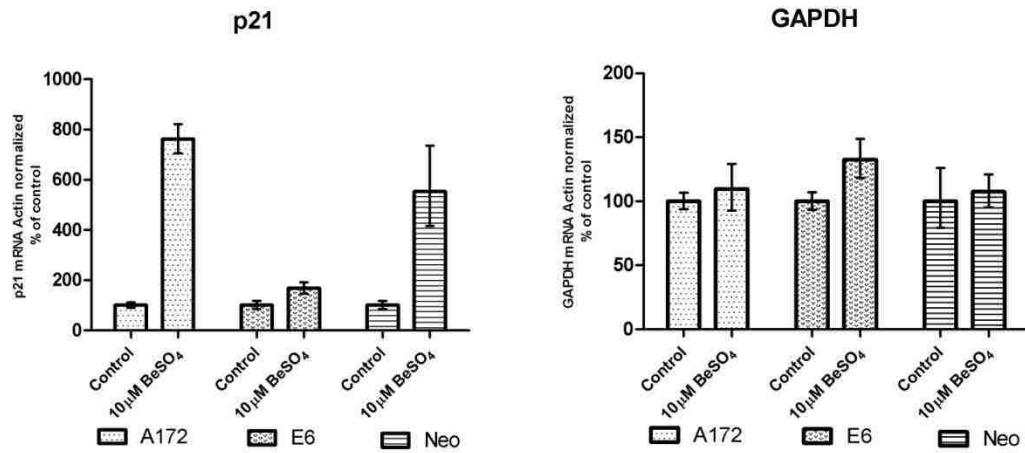


Figure 5-5 BeSO₄ requires functional p53 for p21 mRNA increase

A172, A172 E6 and A172 Neo cells were treated with 0 or 10 μM BeSO₄ for 48 hours. mRNA levels of p21, Actin and GAPDH in each sample were quantified using RT-PCR. Actin mRNA values were used for normalization.

A172 cells did not increase in response to beryllium treatment, the protein levels were higher (Fig 5.7).

p53 and p21 protein levels were analyzed in cell types generated by the three different methods. The samples with p53 mRNA or protein knocked down showed less p53 protein compared to control and the corresponding samples also showed decreased p21 (Fig 5.7).

Cyclin E1 and E2 levels are inversely related to functional p53 protein levels

The basal level of cyclin E1 and cyclin E2 mRNA in A172-E6 cells was higher than A172 control cells and showed no change of in response to 10 μ M BeSO₄ treatment. On the other hand the A172 cells exposed to 10 μ M beryllium the level of Cyclin E2 dropped to 8.9% of A172 controls while in A172-neo it decreased to 20.7% of A172 control. Similar trend was observed for cyclin E1 with A172 control cells expressing lower mRNA at 44.9% and neo cell type with 45.8% of A172 control when exposed to 10 μ M beryllium.

Looking at protein levels using western blot, we observed little cyclin E2 in A172 cells. Therefore we investigated other cell types with increased cyclin E2 expression levels in order to verify that cyclin E2 changes could be measured in our system. MCF7 cells expressed detectable levels of both cyclin E1 and E2. Helmbold et al (Helmbold et al., 2009) showed that untreated MCF7 cells express low levels of cyclin E1 and that cyclin E1 levels increase dramatically 7 days after ionizing radiation (20 Gy) treatment or doxorubicin (1 μ M) treatment. A small increase of cyclin E1 protein was observed at the end of 2 days in ionizing radiation treated samples compared to control cells that received no drug treatment (Fig 5.8a). MCF cell line was used to ensure that we could detect both cyclin E1 and E2. Cyclin E1 was not detectable in A172 and young HFL-1 cells (Fig 5.8b). p53 knock down samples did not show any changes in cyclin E2 protein even at 100 μ M dose. However, A172 and LVp53KO control cells showed

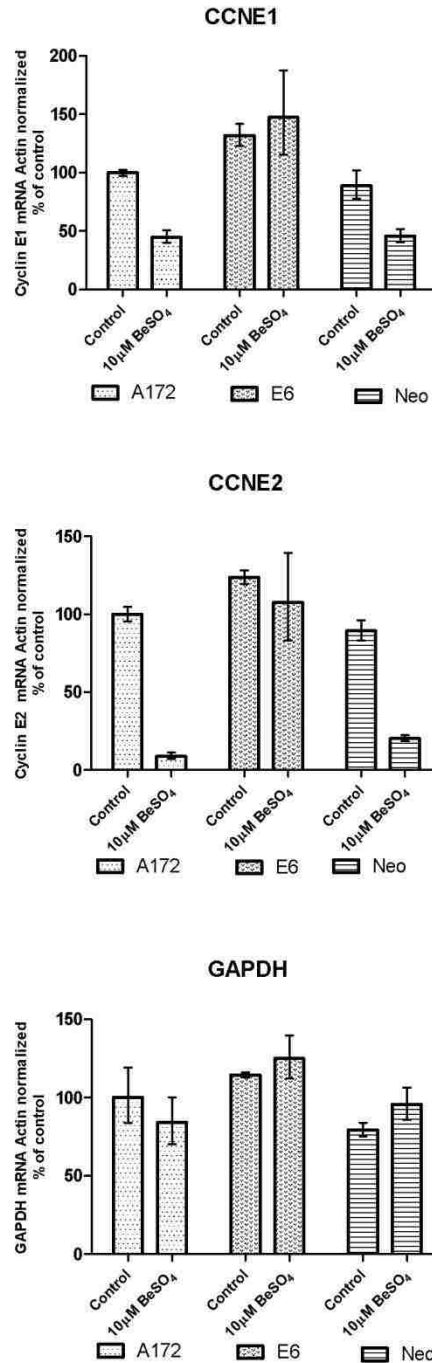


Figure 5-6 Cyclin E1 and E2 mRNA levels are lower in cells with high p53 protein

A172, A172 E6 and A172 Neo cells were treated with 0 or 10 μM BeSO₄ for 48 hours. mRNA levels of cell cycle regulating proteins Cyclin E1 (CCNE1), Cyclin E2 (CCNE2) and housekeeping genes Actin, GAPDH in each sample were measured using RT-PCR. Data are expressed as percent of A172 control mean +/- SD

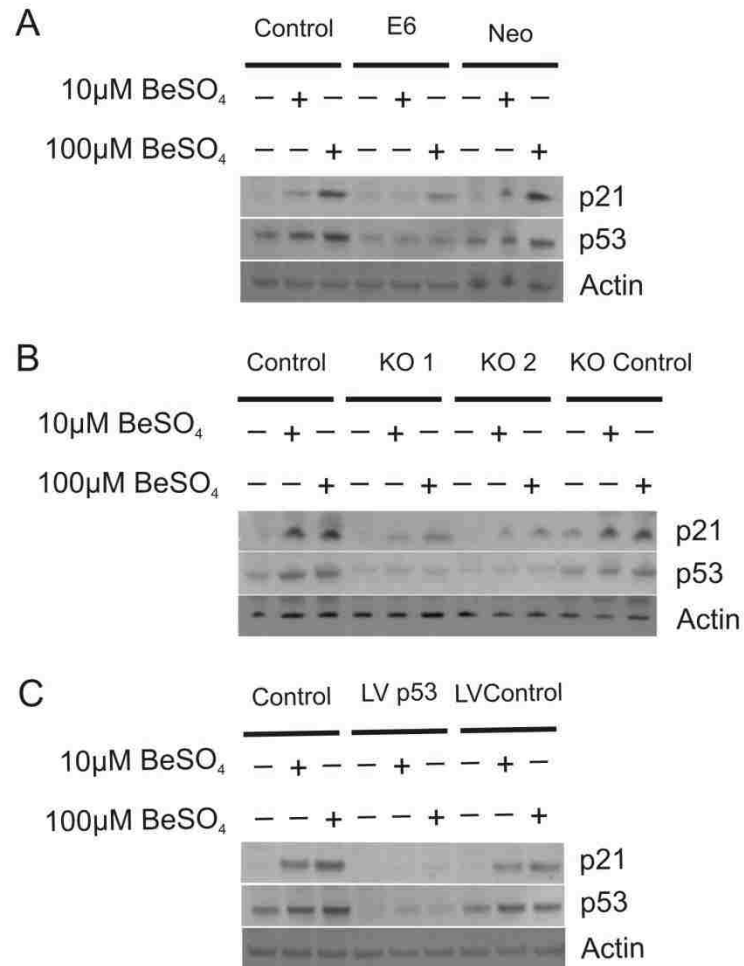


Figure 5.7 p21 protein expression is dependent on p53 protein

- Control cells were normal A172 , Neo were transfection control A172 cells and E6 were A172 cells expressing E6 protein that binds to p53 and degrades it.
- KO1 and KO2 represent two different siRNA sequences used to knock down p53. Normal A172 cells were represented by control and KO Control represents siRNA treatment controls
- LVp53 are A172 cells that have shRNA to silence p53 mRNA, transduced by using lentivirus. LV control are transduction controls and Control samples are from regular A172 cells.

The cells were grown with 0 or 10 μ M beryllium for 48 hours and protein levels were analyzed by western blotting. Same amount of protein was loaded to each lane.

In all three types of p53 knocked down A172 cells p21 expression is dependent on functional p53 protein in the cell.

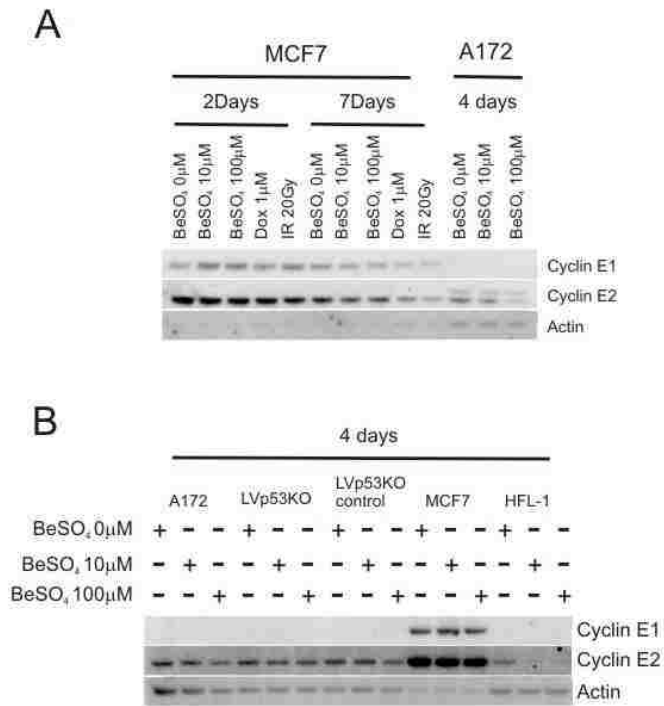


Fig 5.8 Effect of BeSO₄ on Cyclin E2 protein expression is correlated with p53 status in A172 cell line

- To verify if changes in cyclin E could be detected we used MCF7 cell line in an attempt to replicate results from (Helmbold et al., 2009). MCF7 cells were cultured with BeSO₄ at 0, 10 and 100 μM doses in addition to doxorubicin at 1 μM and ionizing radiation at 20Gy. Beryllium was maintained in cell culture during the entire time period. Cells were exposed to doxorubicin for 2 hours. The cells were harvested with MPER at the end of 2 and 7 days. A172 cells were cultured for a period of 4 days in 0,10 and 100 μM doses. A172 cells showed no detectable cyclin E1 but a decrease in cyclin E2 was observed at 100 μM BeSO₄ dose.
- A172 cells, LVp53KO and LVp53KO control cells , MCF7 and HFL-1 young cells were cultured in the presence of BeSO₄ for 4 days and total cell lysate was analyzed for cyclin E1 and E2 proteins. The A172 and LVp53KO control cells showed decrease of cyclin E2 at 100 μM dose. HFL-1 cells showed no detectable cyclin E2 even at 10 μM dose.

decreased cyclin E1 at 100 μ M and HFL-1 cells showed no detectable level of cyclin E2 at both 10 and 100 μ M .

DISCUSSION:

Replicative DNA synthesis occurs during the S phase of cell cycle. BrdU data (Fig 5.3b) shows that there is decrease in DNA synthesis in cells with functional p53 protein when treated with BeSO₄. The decrease in DNA synthesis correlates with the G1 block as evident from cell cycle analysis (Fig 5.3a). Beryllium can cause a cytostatic effect in cells with p53. The cells with p53 knocked down show some resistance to this effect of beryllium (Fig 5.2c).

Be treatment causes phosphorylation of p53 at ser 15 (Gorjala and Gary, 2010). p53 stabilization is correlated with the phosphorylation of Ser 15 (Shieh et al., 1997). In the presence of beryllium p53 binds to p21 promoter region (Coates et al., 2007). However, p21 can also be induced in a p53 independent manner (Michieli et al., 1994). Therefore we sought to determine whether p53 is necessary for p21 induction by beryllium salt.

Here we report that p21 induction by BeSO₄ has been found to be p53 dependent (Fig 5.4, 5.5, 5.7). We have observed that there is no significant change in p53 mRNA but p53 protein is found to increase in response to beryllium treatment in dose dependent manner (Fig 5.4, 5.7).

RNAi mediated removal of p53 mRNA from cells or inactivation of p53 by E6 expression leads to a significant decrease of p21 gene expression (Fig 5.4, 5.5). Lower p21 mRNA in cells is reflected as lower p21 protein concentration in cells with p53 knocked down. p53 is required for p21 induction in situations where DNA damage is caused by radiation (El-Deiry et al., 1994; Michieli et al., 1994). Beryllium induces p53 and p21 without any DNA damage (Gorjala and Gary, 2010). The data presented here demonstrate that cells dosed with beryllium induce p21 in a p53 dependent manner.

Cyclin E2 is different from cyclin E (now called cyclin E1) in that it follows slightly different pattern of activity during cell cycle (Gudas et al., 1999; Lauper et al., 1998). Cyclin E2 mRNA levels peak at the G1-S transition phase. Normal human foreskin fibroblast cells were found to show increased cyclin E2 mRNA when p53 is degraded by E6 expression in these cell lines (Zariwala et al., 1998). In a microarray study of p53-mediated transcriptional repression, *CCNE2* was identified as one of the 111 genes that were down-regulated at least 2 fold after p53 over expression (Spurgers et al., 2006). Using microarray our group has found that *CCNE2* is downregulated following beryllium treatment (unpublished data). Therefore, we sought to validate this finding using qualitative RT-PCR. If there is a decrease in expression of *CCNE2* in response to beryllium we wanted to determine if it is p53-dependent beryllium response.

We have found that A172 cells treated with BeSO_4 with functional p53 show a significant decrease of cyclin E2 mRNA, to a lesser extent cyclin E1. The cells with p53 inactivated show little change in cyclin E1 and E2 mRNA levels. The change of Cyclin E1 and E2 mRNA levels do not correlate with the corresponding protein expression. We found that cyclin E1 is not detectable in the total cell lysates of A172. A172 is glioma cell line and cyclin E1 is absent in human brain cells while cyclin E2 is observed in high levels(Zariwala et al., 1998). Using the same amount of total protein, beryllium resistant MCF7 cells expressed cyclin E1 protein that could be visualized using western blot. In A172 cells the changes in cyclin E2 protein in response to beryllium treatment could be seen after 4 day exposure at 10 and 100 μM (Fig 5.8a, 5.8b). In A172 cells The mRNA changes could be seen at the end of 48 hours upon exposure to 10 μM dose(Fig 5.6). Our experiments with A172 cells at shorter time points did not show observable changes in cyclin E2 protein levels (data not shown). The normal lung fibroblast cells HFL-1 showed decrease of cyclin E2 protein at 10 μM dose for 4 day exposure (Fig 5.8b). BeSO_4 is known to increase p53 protein in HFL-1 cells (Coates et al., 2007).

In the present study we have shown that p53 knock down in A172 is resulted in increased expression of cyclin E2 mRNA and protein. This correlates with E6 expressing cells entering S phase of cell cycle with higher DNA synthesis compared to cells normal A172 with functional p53 (Fig 5.3). All the evidence presents a possibility where BeSO₄ may be activating a branch of p53 network that inhibits cell proliferation via p21 and also simultaneously decreasing the level of cyclin E2 mRNA initially and protein at later stages to induce G1-S block.

CHAPTER 6 BERYLLIUM INDUCED CASPASE 3/7 DOES NOT CAUSE APOPTOSIS

INTRODUCTION

p53 is an important determinant in cellular response to stress. The apoptosis inducing function of p53 is under intensive study and its transcription dependent and independent pathways to induce apoptosis have been identified. The extrinsic pathway uses death receptors of tumor necrosis factor family of receptors (TNF-R) (Ashkenazi and Dixit, 1998). Activation of these receptors leads to cascade of events resulting in inducing activity of caspase 3, which in turn commits the cell to apoptosis. On the other hand p53 dependent intrinsic pathway is activated in the event of DNA damage and results in release of cytochrome c from mitochondria. cytochrome c forms a complex with apoptotic protease-activating factor 1 and procaspase 9 to activate other executioner caspases like caspase 3 (Gross et al., 1999; Li et al., 1998; Nicholson and Thornberry, 2003).

The extrinsic pathway for apoptosis is activated by p53 dependent transcription of genes for Fas, DR5 and PERP. Among these Fas is an important member of TNF-R family of receptors that plays a crucial role in extrinsic pathway. p53 transcription factor binds to promoter of the Fas gene there by inducing its mRNA expression (Muller et al., 1998). Unpublished data from microarray experiment on HFL-1 young cells treated with and without beryllium showed that some genes related to apoptosis are activated. FAS gene is the most up regulated in the list of genes in Table 6.1 (unpublished data, data not shown). One of the potential therapeutic strategies used in treatment of cancer involves using Fas and Fas ligand which are members of death receptor pathways.

Here we present data that shows that caspase activation due to beryllium may

NCBI Reference Sequence	Gene	Definition
NM_001065	TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A
NM_001188	BAK1	BCL2-antagonist/killer 1
NM_003842	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
NM_005921	MAP3K1	mitogen-activated protein kinase kinase kinase 1
NM_138578	BCL2L1	BCL2-like 1
NM_033292	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
NM_000043	FAS	Fas (TNF receptor superfamily, member 6)

Table 6-1 Apoptotic genes upregulated in beryllium treated HFL-1 cells

List of genes up-regulated in HFL-1(Y) treated +/- 10µM beryllium in microarray analysis (unpublished microarray data from Gary Lab; gene expression changes have not yet been evaluated by RT-PCR for verification).

not be leading to apoptosis. BrdU wash out experiment data shows that cells can revert back to active DNA synthesis state after the beryllium is removed from media. The data from XTT assays demonstrates that mitochondrial function is intact in the presence of beryllium. However, from the cell count data it is observed that there is decrease in cell number over long periods of exposure to higher concentration of beryllium. The

apoptosis signals initiated by death receptor pathways converge on the effector caspases like caspase 3 (Ashkenazi and Dixit, 1998; Schulze-Osthoff et al., 1998). Caspase 3 and 7 that are crucial for execution of apoptosis are activated in both extrinsic or receptor mediated and intrinsic or non-receptor mediated apoptotic pathway (Elmore S, 2007). The results documented in this chapter are from experiments setup in an attempt to verify if there is any apoptosis due to caspase 3/7 activity in the presence of beryllium.

MATERIALS AND METHODS:

Cell Culture

HFL-1 (human lung fibroblasts), Wild type A172 (human glioblastoma) and RKO (human colon carcinoma) cells obtained from ATCC, were used for experiments. The cell lines were propagated RPMI1640 with GlutaMAX supplemented with 10% fetal bovine serum and 1% PSF. The cells were cultured in 100mm dishes at 37°C with 5% CO₂. 0.05% trypsin EDTA was used for trypsinizing the cells when splitting. KO1 and KO2 – p53 knock down was achieved using siRNA as described in chapter 5.

Drug treatment:

BeSO₄.4H₂O from Fluka was used to prepare a stock solution. Beryllium stock solution was diluted to required concentration in RPMI just before dosing. Staurosporine was from Calbiochem (Cat# 569396) and Sigma (Cat# S6942). Camptothecin from Sigma (Cat #C9911) was prepared according to instructions by dissolving 10mg in 1mL DMSO. Etoposide from Sigma (Prod. No. E1383) was prepared as a 50mM stock according to instructions. Q-VD-OPH (Cat#OPH 109) was purchased from Enzyme systems products Solon, Ohio.

Caspase assay

A predetermined number of cells are seeded into 96 well plate and cultured with or without drug. Activity of caspase 3/7 was measured with Caspase Glo 3/7 Assay (Cat

#G0890) and ApoTox-Glo™ Triplex Assay (Cat #G6321) from promega. When 384 well plate is used for assay the cells are cultured in 60mm plates with or without drug for the required length of time. The cells are then trypsinized and a specific volume from each plate is transferred to 384 well plates in triplicate.

TUNEL assay

Was performed with Fluorescein-12-dUTP based method optimized in house. The cells were analyzed using Flowcytometry. Detailed protocol adapted from original protocol by Heatwole VM (Heatwole, 1999) is given in appendix VI.

DNA synthesis, Metabolism and Cell number

Cells were grown in RPMI in the presence of required concentrations of BeSO₄ on 100mm plates. Each dose was setup in triplicate. After 64 hours cell were trypsinized and seeded into a 96well plate at the rate of 26000 cells per well. Cells from each plate were seeded in triplicates. Three identical plates were setup. Remaining cells were seeded into new 100mm plates for day 6 experiment. The cells in 96 well plate were allowed to attach for additional 8 hours. At the end of 72 hours cells were washed and BrdU incorporation assay was performed according to kit instructions from EMD Biosciences/Calbiochem. Similarly plates for XTT assay and CyQuant cell proliferation assay were also performed on the respective plates.

Statistical Analysis

Data sets with two dosage groups at each time point were analyzed by unpaired t-test, two tailed. Data sets with more than two groups were analyzed using one-way ANOVA with a post-hoc Bonferroni's multiple comparison test (P < 0.05 considered significant).

RESULTS

Staurosporine, Camptothecin and Etoposide induce caspase 3/7 in A172 and RKO

Both A172 and RKO cell lines show increase in caspase 3/7 activity in response to treatment with apoptosis inducing drugs staurosporine, camptothecin and etoposide. The major difference in response between the cell lines is that RKO shows huge increase of caspase 3/7 at early time points of 12 and 24 hours whereas the increase in A172 cell line is at later time points of 24 and 48 hours (Fig 6.1).

In A172 cells staurosporine was able to produce an increase caspase3/7 activity of 421% of control in first 12 hours which further increased with time reaching upto 1029% of control by the end of 48 hours. Camptothecin and etoposide however showed caspase3/7 activity of approximately twice the control values at 24 hour time point which increased to 743% and 530% of control by 48 hour time point.

RKO cells show an opposite pattern to A172 with highest activity of caspase 3/7 in first 12 hours by staurosporine at 871% of control which decreased to 232% of control by 48 hour time point. Camptothecin used at 10 μ M concentration induced caspase 3/7 within first 12 hours and is maintained approximately at the same level even at the end of 48 hour time. Etoposide induced caspase 3/7 in RKO cells in a manner that is different from the other two drugs showing a very small increase of 150% of control in first 12 hours followed by a maximum increase upto 618% of control by 24 hours which is maintained at 537% by the end of 48 hour time point.

Beryllium induces caspase 3/7 in A172 but not RKO

Response of the two cell lines A172 and RKO to staurosporine, camptothecin and etoposide is almost same as in Fig 6.1. RKO showed no change of caspase 3/7 activity in response to beryllium treatment both at 24 and 48 hours (Fig 6.2). BeSO₄ was able to induce a dose dependent increase of caspase 3/7 at 48hrs in A172 cells. A 10 μ M dose of beryllium was able to induce 303% of control increase of activity of caspase

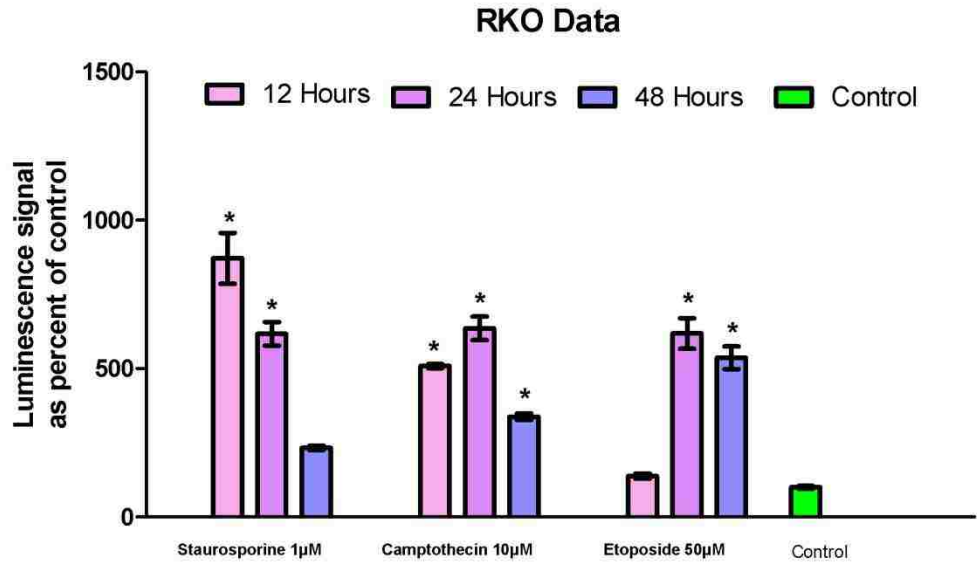
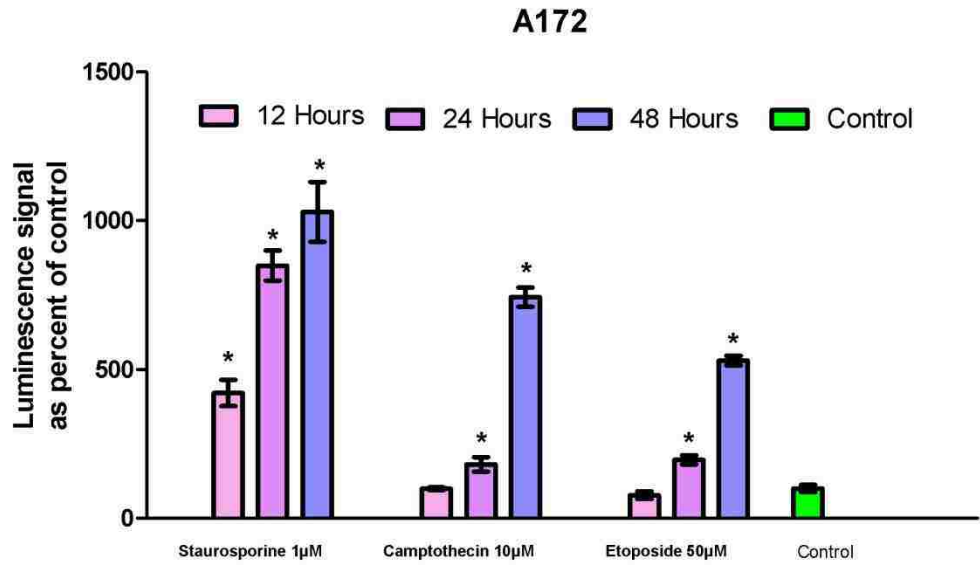


Figure 6-1 Staurosporine, Camptothecin and Etoposide induce caspase 3/7 in both A172 and RKO

2500cells of A172 and 2000 cells of RKO were seeded into each well of a 96well plate. Each dose was seeded in triplicate. Media with staurosporin at 1µM, camptothecin at 10µM and etoposide at 50µM was supplied at 48hrs, 24hrs and 12hrs before the assay time point. Caspase 3/7 activity was measured using Caspase Glo assay from promega. Apoptosis inducing drugs staurosporin, camptothecin and etoposide cause increase of caspase of approximately same amount in both A172 and RKO cell lines but at different time points. The data are presented as mean +/- Standard Deviation. Statistically significant differences are indicated with * $P < 0.05$.

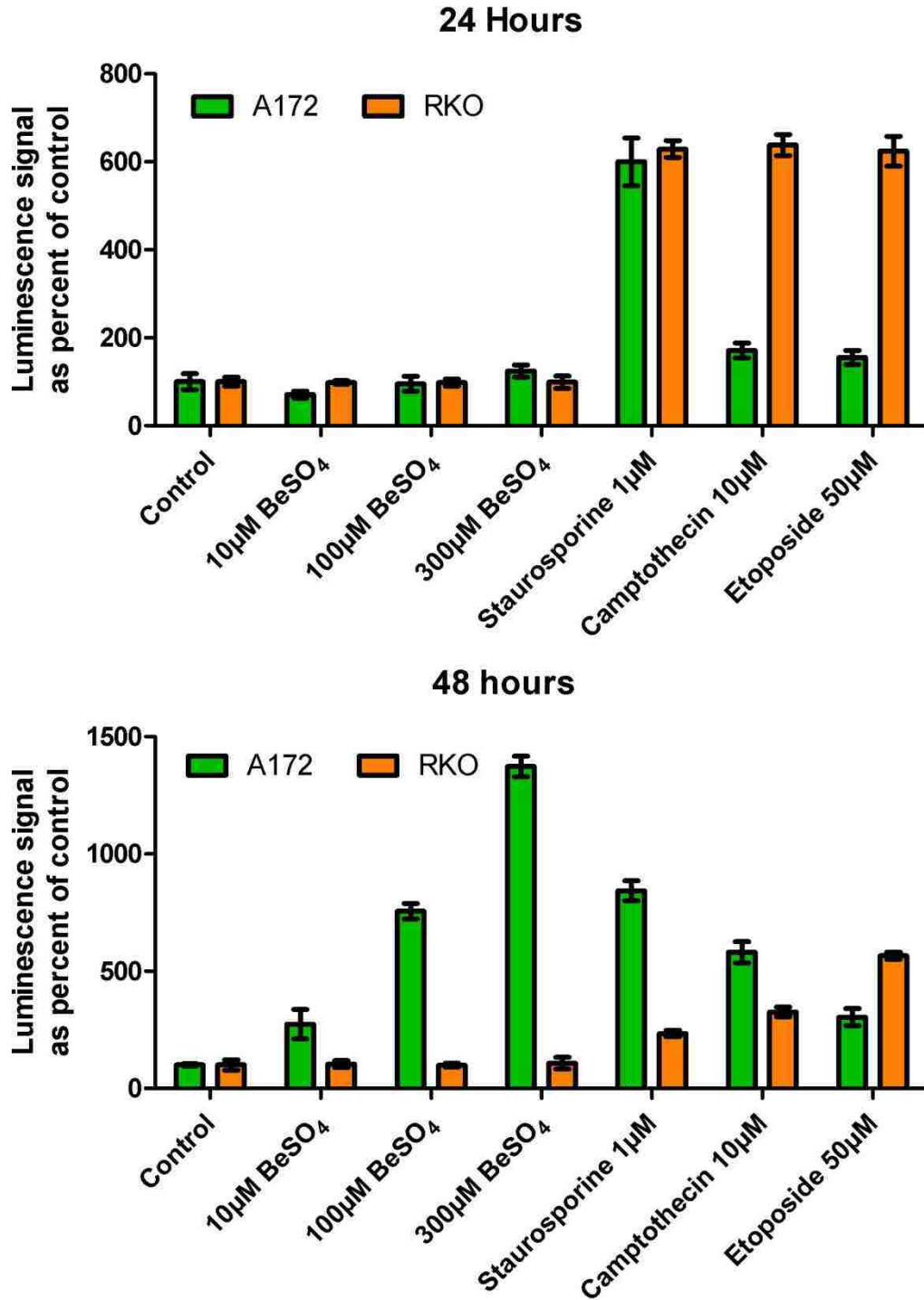


Figure 6-2 Beryllium induces caspase in A172 but not RKO

2500 cells of A172 and 2000 cells of RKO were seeded into the wells of a 96 well plate. Every dose was seeded in triplicate. Media +/- drug was supplied at 48hrs and 24hrs before the assay time point. The data are presented as mean +/- Standard Deviation.

3/7 which is comparable to effect of 50 μ M dose etoposide at 347% of control. 100 μ M and 300 μ M doses of beryllium increased activity of caspase3/7 to 832% and 1484% of control. 1 μ M staurosporine and 10 μ M camptothecin caused an increase of 915% and 625% of control respectively.

Beryllium can induce caspase 3/7 in A172 and HFL-1(Y) but not in HFL-1 (S)

Both at 24 and 48hr time point beryllium was not able to induce caspase in HFL-1(S) cells (Fig 6.3). In rapidly dividing A172 and HFL-1(Y) cells beryllium induced caspase3/7 in dose dependent manner at 48hrs. The total amount of caspase3/7 induced in A172 is higher compared to HFL-1(Y).

Functional p53 is required for beryllium to induce caspase3/7

From the results (Fig 6.4) we can see that KO1 cell type has abnormally high caspase3/7 activity. This may be caused by the duplex oligo used in siRNA treatment for p53 knock down. It should be noted that the morphology of KO1 cells was also abnormal compared to A172, KO1 or KO control.

As seen in earlier experiments the A172 cells show dose dependent increase of caspase3/7 activity. The KO control cell type also shows similar pattern. The KO2 cell type with p53 knock down shows almost no increase of caspase3/7. At 300 μ M dose, it can be seen that there is difference in activity of caspase3/7 between A172 control (587.5%), KO control (479.8%) and KO2 (265.6%). This data suggests a possibility that functional p53 may be required for beryllium based increase of caspase activity.

Beryllium induced caspase3/7 may not cause cell death in A172 and HFL-1(Y) cells

Q-VD-OPH is an anti-apoptotic drug that can inhibit activity of many different caspases including caspase 3. The results (Fig 6.5) demonstrate that Q-VD-OPH at 50 μ M concentration was able to inhibit activation of caspase induced by beryllium even at a high dose of 100 μ M. The response of both A172 and HFL-1(Y) cells is following the same pattern.

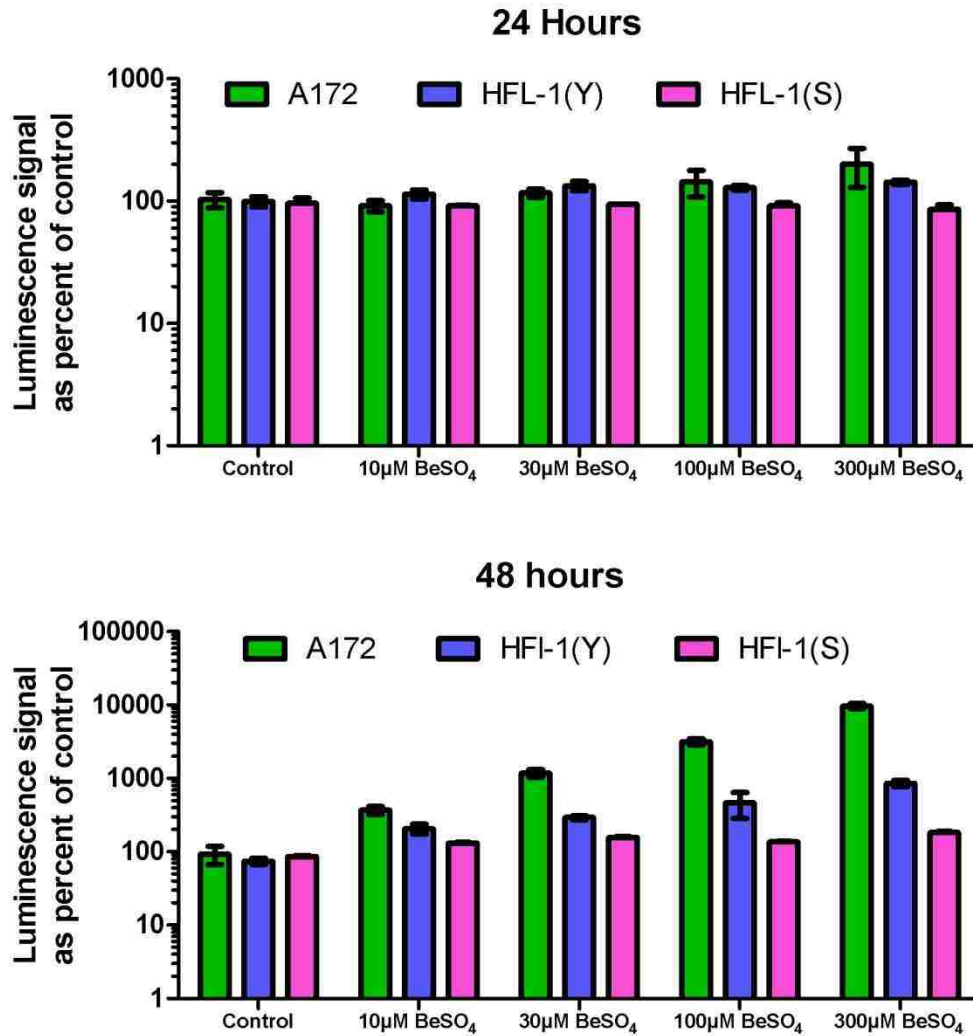


Figure 6-3 Beryllium can induce caspase 3/7 in A172 and HFL-1(Y) but not in HFL-1 (S)

2500cells of A172 and 2000 cells of HFL-1(Y) at passage 14 were seeded into each well of a 96well plate. HFL-1 (S) cells at passage 33, that are not dividing were seeded at 16,000 cells per well. Each cell line for each dose was seeded in triplicate. Media +/- drug was supplied at 48hrs and 24hrs before the assay time point. The results are plotted as percent of control on log scale to present the differences between cell lines clearly. The data are presented as mean +/- Standard Deviation.

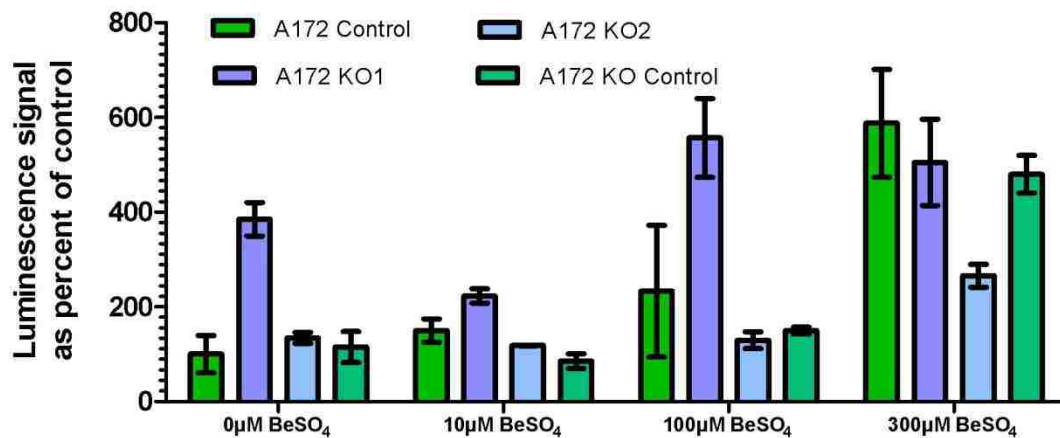


Figure 6-4 Functional p53 is required for beryllium to induce caspase3/7

A172 cells treated with siRNA to knock down p53, KO1 and KO2 along with KO control and regular A172 cells were cultured in 60mm plates with +/- beryllium. Each dose for each cell type setup in triplicates. At the end of 48hrs, 20μL of the cell suspension from these plates(including the floating cells) was seeded into 384well plate. Contents from each plate seeded to 3 wells. ApoTox-Glo™ Triplex Assay was used to generate this data. This kit combines caspase glo kit with a Live / Dead cell detection assay. This enables measuring of 3 parameters in each well. The caspase values were normalized with ratio of live cell to dead cells. All values expressed as percent of Control at 0μM beryllium. Abnormally high caspase 3/7 in KO1 0μM may be caused by the duplex oligo used in siRNA treatment. Unlike KO2 and KO control. The data are presented as mean +/- Standard Deviation.

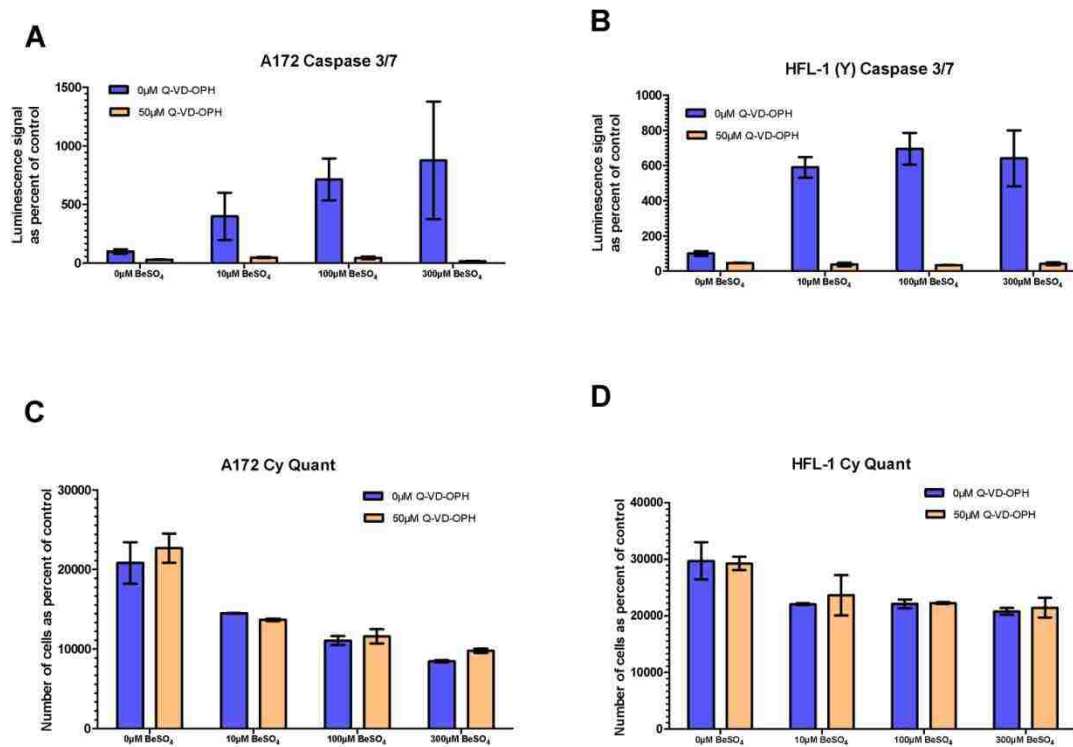


Figure 6-5 Beryllium induced caspase can be inhibited by QVDOPH

6.5a and b: 2000 cells of A172 and 2500 cells of HFL-1(Y) were seeded into each well of 96well plate. Two 96 well plates were setup for each cell line. The cells were dosed with RPMI +/- beryllium and beryllium + Q-VD-OPH (50μM). At the end of 48hrs cy-quant and caspase assay were performed on the respective plates. The data are presented as mean +/- Standard Deviation.

In the presence of beryllium alone or beryllium and Q-VD-OPH we can see that there is decrease of cell number with increasing concentration of beryllium. The cell number measurement was done using Cy-Quant assay.

Taken together these results show that the decrease in cell number in the presence of beryllium may not be due to apoptosis caused by caspase3/7 activation.

Beryllium induced caspase does not lead to Apoptosis

Beryllium at 10 μM did not cause any labeling by TUNEL assay as compared to control cells which did not receive any drug treatment (Fig 6.6). At a higher concentration of 300 μM there is around 10% higher signal compared to control. On the other hand the positive controls DNAase I treated cells (39%), camptothecin (10 μM – 67% and 100 μM – 86%) and staurosporine (69%) treated cells show a very high percentage of cells undergoing apoptosis as detected by increase in signal. The difference in TUNEL positive signal between beryllium treated and positive control treatments indicate that beryllium may not be inducing apoptosis to the extent as expected by the caspase3/7 activation.

Beryllium induced inhibition of DNA synthesis is reversible

In the presence of 10 μM BeSO_4 by the end of 72 hours there is significant decrease of DNA synthesis as measured by BrdU incorporation assay (Fig 6.7A). For the same time point and dose, the activity of mitochondria (Fig 6.7B) or the total DNA content (Fig 6.7C) did not change comparably. However, when the cells are continued in culture without beryllium the DNA synthesis activity showed recovery reaching 50% of control treatment by the end of 6 days and by 9 days the recovery was almost 100% of control (Fig 6.7A). There is no change in mitochondrial activity and total DNA content at day 6 and 9 (Fig 6.7B, 6.7C).

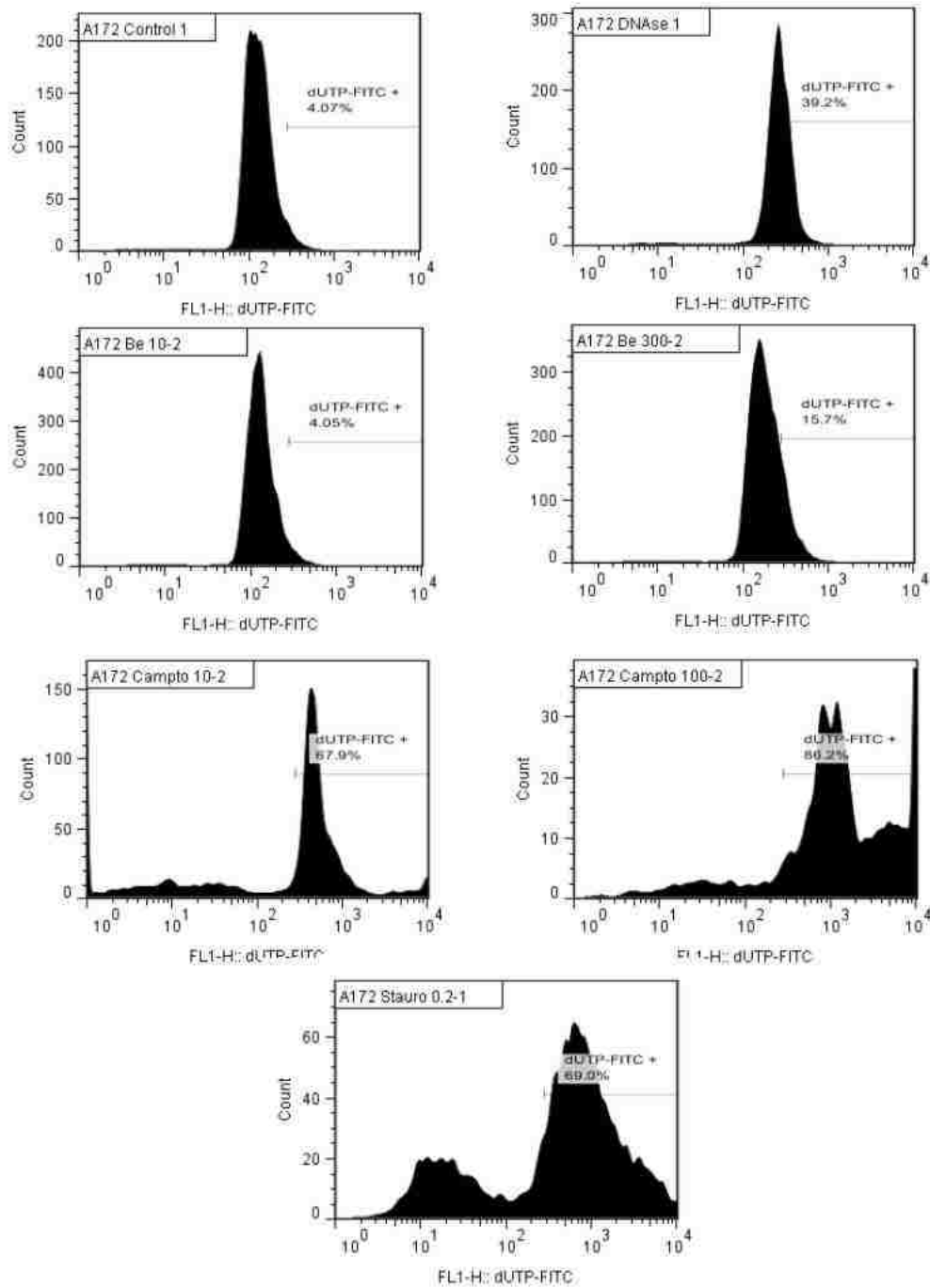


Figure 6-6 Beryllium induced caspase does not lead to Apoptosis

A172 cells were treated with 10 μ M and 100 μ M beryllium for 48hours, Camptothecin 10 μ M and 100 μ M for 48 hours and staurosporine 0.2 μ M for 24 hours were processed according to TUNEL assay protocol for flowcyotmetry. The data are presented as mean +/- Standard Deviation.

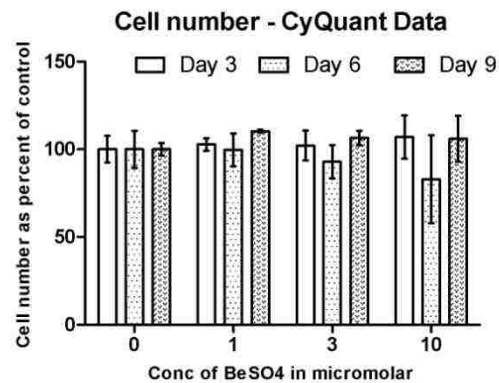
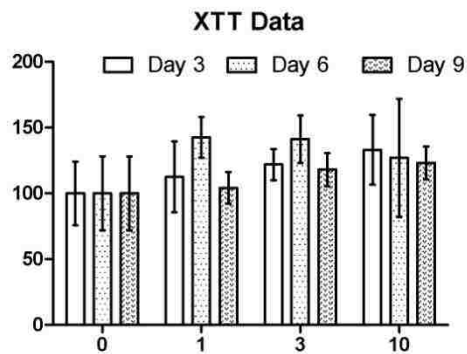
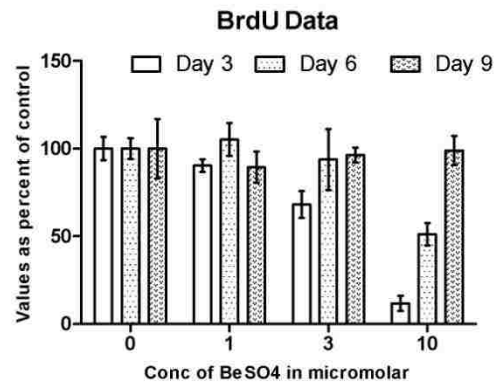


Figure 6-7 Beryllium induced inhibition of DNA synthesis is reversible

A172 cells were treated with 0μM, 1μM, 3μM and 10μM beryllium for 72hours as described in methods section and day 3 readings were taken for BrdU incorporation, XTT and CyQuant. The cells were cultured further in RPMI without beryllium and readings were taken on day 6 and day 9. The data are presented as mean +/- Standard Deviation.

DISCUSSION

The data in chapter 4 shows that beryllium may not be causing cell death. The microarray data showed increased expression of apoptosis related genes. Due to this the possibility of apoptosis had to be investigated. To use as positive controls we have selected 3 drugs (staurosporine, camptothecin and etoposide) that are known to induce apoptosis.

The exact mechanism of action of staurosporine, a protein kinase inhibitor, is not clearly understood. But studies point to a possibility that it can induce apoptosis at early stages through caspase-dependent mechanism and at later stages can employ a separate signaling pathway that is caspase-independent (Belmokhtar et al., 2001; Zhang et al., 2004). Camptothecin forms a complex with DNA and topoisomerase I resulting in DNA damage. It is specifically potent in S phase cells (Hsiang et al., 1985; Hsiang et al., 1989). Etoposide is a DNA topoisomerase II inhibitor and functions by blocking DNA topoisomerase resulting in DNA strand break. Etoposide requires p53 for its apoptosis inducing function (Grandela et al., 2007; Karpinich et al., 2002). The time points at which these drugs induce activity of caspase may be different from each other due to difference in mechanism of action. The same reason may also explain the difference in the time points of caspase induction between cell lines. However, the three drugs were able to induce caspase 3/7 activity in both the sensitive A172 and the resistant RKO cell line. The apoptosis inducing effect of DNA replication interfering drugs, camptothecin and etoposide, as measured by caspase3/7 activity is seen at earlier time point in RKO cells compared to A172. This may be due the shorter cell doubling time of RKO cell line.

The standard apoptosis inducing drugs were able to induce caspase in RKO cell line within 24 hours, BeSO₄ did not show comparable effect on this beryllium resistant cell line. The caspase 3/7 activity was dose dependent in A172 cell line and is seen at 48 hour time point. The timing of caspase 3/7 activity induction is similar to camptothecin

and etoposide suggesting that beryllium may be following the similar pathway used by these two drugs. However it has to be noted that in chapter 4 it was demonstrated that there is no DNA damage as seen by phosphorylation of γ -H2AX. The caspase 3/7 activity measured in the presence of BeSO₄ in HFL-1 young (Y) and senescent (S) cell lines (Fig 6.3) suggests that active cell division is required for BeSO₄ to induce caspase3/7 activity. Additionally the BrdU incorporation assay on A172 cells shows that beryllium induced inhibition of DNA synthesis is not permanent (Fig 6.7A). Also there is no decrease in metabolic activity of cells in the presence of beryllium as seen from the XTT data (Fig 6.7B).

TNF receptor superfamily member, Fas gene is upregulated by beryllium dosing as seen in preliminary data from microarray experiment. Evidence from other studies that fas gene can be upregulated by p53 (Muller et al., 1998). Apoptosis initiated by death receptor through extrinsic pathway also results in activation of caspase 3/7. Caspase3/7 activity measured in p53 knocked down cells provides some evidence that functional p53 may be required for activation of caspase3/7, especially at higher doses of 100 and 300 μ M beryllium. In the siRNA system used for this experiment one of the duplex oligos used to generate KO cell type KO1 may be causing activation of caspase 3/7.

The distinct feature of apoptosis is that the initiation of caspase cascade results in DNA fragmentation. In any case if only caspase initiation is observed without any sign of other characteristic features of apoptosis like DNA fragmentation such a cell death cannot qualify as apoptosis (Takemura et al., 2001). The TdT-mediated dUTP Nick End Labeling assay called as TUNEL assay, labels the ends of the degrading DNA in apoptotic cells using the enzymatic activity of polymerase terminal deoxynucleotidyl transferase (TdT) (Gorczyca et al., 1992; Gorczyca et al., 1993). The flowcytometry

based TUNEL data shows that beryllium at 10 μ M does not cause any labeling. The data suggests that DNA fragmentation is not taking place in the presence of beryllium.

Recent discoveries show that there are caspase-independent mechanisms that can cause apoptosis. Discovery of XIAP based inhibition of caspase 3 showed that activation of caspase 3 does not necessarily always induce apoptosis (Deveraux et al., 1999; Scott et al., 2005). Studies have shown that XIAP based caspase 3 inhibition protects the cell from CD95 (Fas/Apo-1) mediated apoptosis (Bratton et al., 2002). We have used a well-known pan caspase inhibitor Q-VD-OPH to verify if the reduction in cell number seen at higher doses of beryllium is due to cell death caused by caspase induction. Q-VD-OPH has antiapoptotic properties and is not toxic to cells high concentration (Caserta et al., 2003). From the data (Fig 6.5 A, 6.5B) it is evident that 50 μ M Q-VD-OPH was able to effectively inhibit the activity of caspase 3/7 at all concentrations of beryllium used in the study. The cyquant assay data used to calculate cell numbers on a replicate plate setup in parallel shows that the Q-VD-OPH inhibition of caspase 3/7 is not able to rescue the cells from cell death at higher concentrations of beryllium (Fig 6.5C, 6.5D).

The scope of this study is limited to check if the increase in caspase 3/7 is resulting in apoptosis of beryllium sensitive cell line. In this study we have checked an early and a late markers of apoptosis i.e., caspase 3/7 activity and DNA fragmentation. These markers give information concerned with nuclear and cytosolic compartments of the cell. To completely evaluate the effect of beryllium with respect to apoptosis further study is required using RT-PCR and western blotting techniques to evaluate the expression of mRNA and proteins of early markers like bcl-1, BAX, TRAIL. These proteins are concerned with changes in mitochondria. Flow cytometry based evaluation of membrane changes using Annexin V and mRNA, protein data related to Fas receptor activity will give a complete picture.

CHAPTER 7 SUMMARY AND FUTURE DIRECTIONS

SUMMARY

1. Beryllium salt can inhibit cell proliferation in susceptible cancer cell lines.
2. BeSO₄ causes phosphorylation of p53. This stabilizes p53 leading to its accumulation in the cell.
3. Other changes induced by BeSO₄ treatment include increase of p21, Senescence-associated beta-galactosidase activity. G1-S cell cycle block and decreased DNA synthesis are also observed.
4. In spite of changes in intracellular activity that resembles DNA damage response pathway there no DNA damage caused by BeSO₄.
5. Increase of p21 and cell cycle arrest in response to BeSO₄ treatment are p53 dependent.
6. p53 is required in sensitive cancer cells for regulating cyclin E2 expression in response to BeSO₄ treatment.
7. Caspase 3/7 activity was found to be increased in beryllium sensitive cancer cells treated with BeSO₄. But there is no correlation between caspase 3/7 activity to number of cells undergoing apoptosis.

FUTURE DIRECTION

1. Survey of cancer cell lines in chapter 3 revealed that different cell lines have different levels of tolerance to BeSO₄ dosing. ICP-MS data in chapter 4 shows that the uptake of beryllium by sensitive and resistant cell types is almost equal thus, suggesting that the amount of beryllium inside the cells may not be the reason for differences in sensitivity to BeSO₄. We have also found that functional p53 is required for induction of cell proliferation arrest in response to BeSO₄ treatment. The survey included wild type and mutant p53 expressing cell lines. No correlation was observed between p53 status and beryllium resistance. The mechanism by which some cell lines can resist beryllium

is not clear. A detailed survey which includes a wide range of cell types from different tissue sources may help in identification of the pattern.

2. A nominal decrease in mRNA expression of cyclin E1 and E2 is observed in A172 cells by the end of 48 hours of BeSO₄ treatment. Such a change is not seen in the corresponding protein levels by 48 hours (data not shown). However, cyclin E2 protein levels decreased by the end of 4 days. Cyclin E1 protein levels were below detection limit of the antibodies used in western blotting. Cyclin E1 and E2 protein expression begins at the same time during G1-S transition. The peak expression and degradation of cyclin E1 arrive early compared to cyclin E2 (Lauper et al., 1998). Studies have shown that ubiquitin dependent proteolysis ensures early degradation of cyclin E1 (Won K and Reed S., 1996). However, not much research has been done related to cyclin E2.

We have also observed that altered cyclin E2 mRNA levels in beryllium treated cells are dependent on p53. *CCNE2* is one among the cell cycle regulatory genes that is identified as a possible target for miR-34 miRNAs. mir-34 family of miRNAs that are in turn regulated by p53 (He L et al ., 2007). The details of a possible pathway through which p53 dependent regulation of cyclin E2 operates are not yet clearly known. A detailed study to investigate the mechanism that regulates the activity of cyclin E2 through p53 will be contribute to overall understanding of the cell cycle arresting effects of BeSO₄.

3. Preliminary data from microarray experiments showed that *FAS* gene is upregulated in response to BeSO₄ treatment. It has been found that p53 can bind to Fas gene promoter (Munsch et al., 2000). In the present study we have shown that functional p53 is required for growth arresting effects of beryllium. An earlier study published by our lab group has demonstrated that in response to BeSO₄ treatment p53 functions like a transcription factor, binds to the promoter region and induces p21 mRNA expression (Coates SSA et al., 2007). Validation of microarray results related to *FAS* gene

upregulation, followed by gene expression studies using p53 knock out varieties of cell lines will allow us to test whether *FAS* gene expression in the presence of BeSO₄ is also p53 dependent. We have also observed an increased caspase 3/7 activity in response to BeSO₄ treatment. The pathway leading to increase in caspase 3/7 activity in BeSO₄ treated sensitive cell lines is not yet mapped. Since the protein encoded by *FAS* gene plays an important role in extrinsic pathway for apoptosis further investigation into the *FAS* gene related results from microarray data will be a good starting point in elucidating the caspase 3/7 activation pathway.

4. Increase of caspase3/7 did not induce apoptosis in BeSO₄ treated samples. In this study, changes in levels of activated caspase3/7 have been verified using Caspase-Glo 3/7 assay only. Additional assays comparing the total caspase 3 to the amount of activated caspase in the presence and absence of BeSO₄ are required. These assays will help getting an accurate picture and also to validate the results from Caspase-Glo 3/7 Assay.

It may be possible that inhibitor of apoptosis proteins (IAP's) are involved in protecting the cells from caspase 3/7 in the presence of BeSO₄. Some studies have shown that in p53 mediated apoptosis, the increase of p53 protein correlates with decrease of XIAP, a potent member of IAP family (Mohapatra et al., 2005; Zou et al., 2012). An investigation directed towards the activity of IAPs in BeSO₄ treated samples may provide an answer to the failure of apoptosis in the presence of activated caspase 3/7.

Appendix I Characterization of an alternative +/- p53 human glioma model system (U87MG)

BACKGROUND

A172, U87MG cell lines and their corresponding variants transfected to express E6 protein and transfection control sequence were obtained from Dr. Gregory Cairncross, University of Calgary, Alberta T2N 1N4, Canada (Xu et al., 2005). Both cell lines were tested for sensitivity to beryllium using cell count method. A172 cell line data is used in chapter 5. U87MG cell line data is documented here.

EXPERIMENT 1

Objective

To check the sensitivity of U87MG, U87MG-E6 and U87MG-Neo cell lines' to beryllium.

Method

The cells are exposed to selected range of beryllium concentrations at 0, 10,30,100,300 micro molar range for a period of 9 days. The cell counts are taken at the end of 3rd, 6th, and 9th day. Cells were diluted and reseeded to maintain in sub-confluent state.

The initial cell number to be seeded is 90,000 cells/plate. It is known from earlier experiments that U87MG cell line grows slow so higher cell number is seeded instead of regular 75000 cells/plate at 3ml per plate for this experiment. After the cells are allowed to attach for a period of 24 hours, the plates are shuffled, labeled with concentration fo beryllium and +/- beryllium RPMI is supplied to cells. One set of plates seeded for Day 0 counts are trypsinized and counted to get the starting cell number.

Cell count and reseeding procedure

Old media is removed and cells are washed with P.B.S one time.

0.5 mL of trypsin EDTA is added to the cells and plate is incubated for 3 minutes at 37°C. 2.5 mL of RPMI is added to inactivate the trypsin and cells are suspended in RPMI. 100 µL of cell suspension is transferred into a coulter counter cup filled with 9.9 mL of isotone.

7 mL of RPMI with 8/7times the required concentration of BeSO₄ is aliquoted to pre-labeled 15 mL tubes. After taking 100 µL for cell counts, 1 mL of the cell suspension is added to the respective 15 mL tubes with BeSO₄ RPMI, this gives us 1:8 diluted cell suspension of which 3 mL was seeded to new pre-labeled 60 mm dishes.

The above procedure is followed for reseeding and counting cells on day 3,6 and 9. On day 9 experiment is terminated.

Result

From the data obtained it can be seen clearly that U87MG-E6 cells are able to tolerate BeSO₄ presence better compared to the control cell types. This result suggests that functional p53 may be required for the effects of beryllium to be seen (Fig 1).

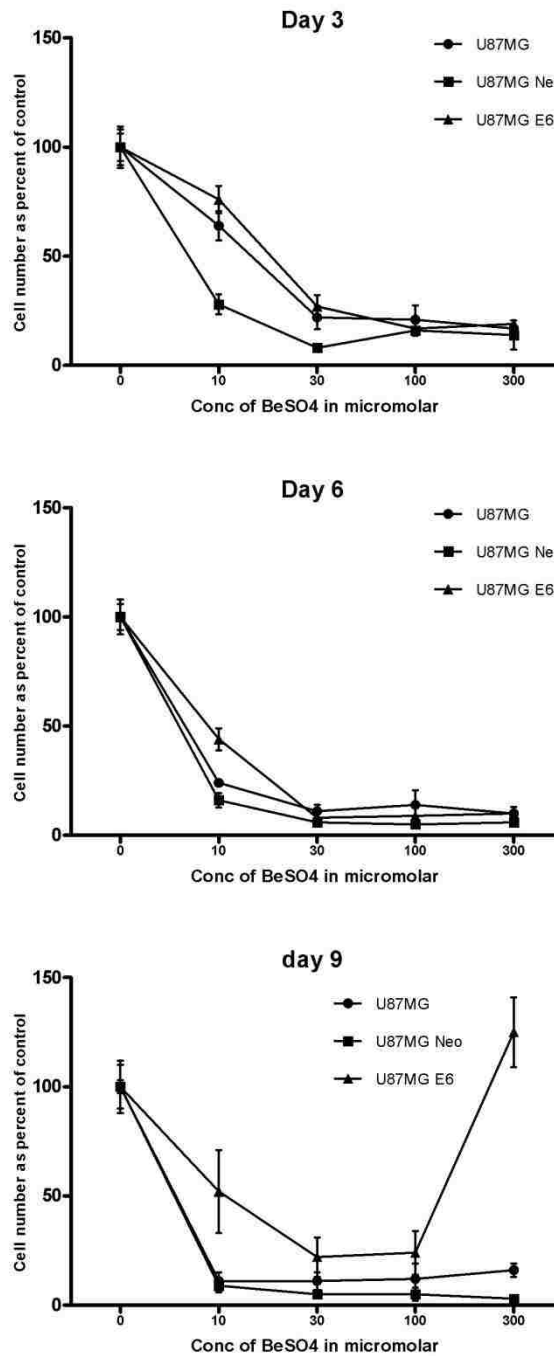


Figure 1 U87MG Cal, Neo and E6 cell counts

Over a period of 9 days U87MG-E6 cell type is able to withstand presence of beryllium compared to Control cell types. The abnormally high reading on day 9 for U87MG-E6 at 300μM concentration is an experimental error. The data are presented as mean +/- Standard Deviation.

EXPERIMENT 2

Objective

To verify the p53 dependence of p21 expression in response to beryllium in U87MG, U87MG-E6 and U87MG-Neo cell lines

Method

All the three cell lines are dosed with 0, 10 μ M and 100 μ M BeSO₄ for 48hours and harvested using MPER with protease and phosphatase inhibitors.

Result

p21 expression is p53 dependent in U87MG cells and is dose dependent in response to BeSO₄ treatment (Fig 2).

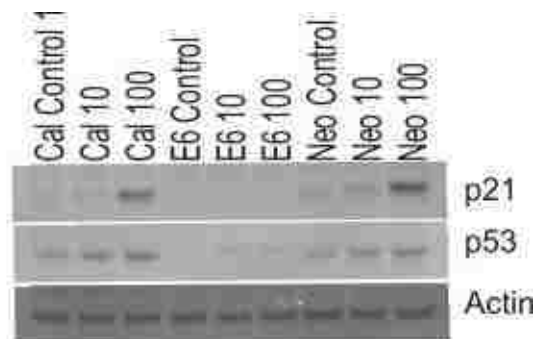


Figure 2 U87MG Cal, Neo and E6 Western blots

Cal cells are regular U87MG, U87MG-E6 express E6 protein that binds to p53 protein making it inactive and U87MG-Neo are transfection control cells.

EXPERIMENT 3

Objective

To verify p53 dependence of p21 gene expression in U87MG cells.

Method

U87MG, U87MG-E6 and U87MG Neo cells were treated with BeSO₄ at 0,10 and 30 μ M BeSO₄ and RNA extraction was done at the end of 48hours.

Result

p21 mRNA expression is p53 dependent in U87MG cells and is dose dependent in response to beryllium treatment (Fig 3)

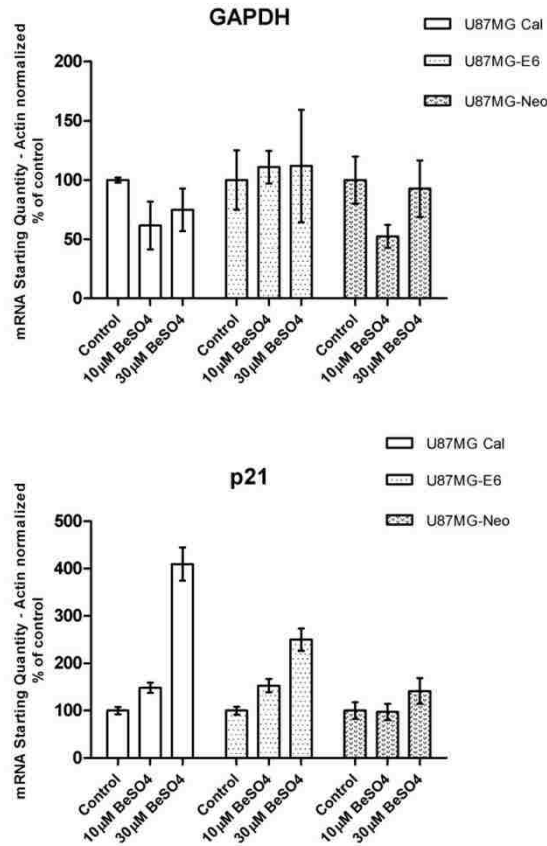


Figure 3 U87MG Cal, Neo and E6 p21 gene expression RT PCR data

Cal cells are regular U87MG, U87MG-E6 express E6 protein that binds to p53 protein making it inactive and U87MG-Neo are transfection control cells. mRNA quantification was done using real-time PCR method. The data are presented as mean +/- Standard Deviation.

References

Xu GW, Mymryk JS, Cairncross JG. (2005) Inactivation of p53 sensitizes astrocytic glioma cells to BCNU and temozolomide, but not cisplatin *J Neurooncol* 74:141-149.

Appendix II Characterization of a spontaneously-arising Be-resistant clonal derivative of A172

BACKGROUND

IsoB11/17RS and IsoB11/9'RS cell types were generated from normal A172 cells by Dr. Ronald Gary. The procedure followed briefly is as follows. Normal A172 cells were grown in Be RPMI for long duration. The surviving cells were propagated resulting in A172 cells that have developed resistance to beryllium. The cell count experiment was done on IsoB11/17RS and IsoB11/9'RS cell lines along with normal A172 cells as controls for comparison.

Objective

To verify if IsoB11/9'RS to beryllium have developed resistance to higher concentrations of beryllium compared to A172 cells.

Method

The cells are to be exposed to 0,1,3,10,30,100,300, 1000, 3000,10,000 μM doses of BeSO_4 for a period of 9 days. The cell counts are taken at 3rd day, 6th day and 9th day. The cells are seeded in 60 mm dishes at 75000 cells/plate with each plate receiving 3 mL cell suspension. Day 3,6,9 cells counting and reseeded is done as described earlier.

Result

- i)** Iso B cell types of A172 are resistant than normal A172 cells (Fig 1)
- ii)** Iso B 11/9'RS appeared to show more resistance to Be than Iso B11/17RS on day 3 but by Day 9 Iso B11/17 was more resistant (Fig 1)
- iii)** Iso B11/17RS appeared to grow faster than Iso B11/9' RS (Fig 2). This is supported by the cell doubling time values and general observation during cell culture
- iv)** Cytostatic concentration (Fig 3)

Cell type	Cytostatic Conc
Iso B 11/17RS	30 μ M
Iso B 11/9'RS	30 μ M
A172 (01/22/07)	10 μ M

Table 1 Cytostatic concentrations for Iso cell types

The lowest conc that is showing same cell number for 3,6,9 day counts is taken as cytostatic (Fig 3). However for all cell lines then next two conc's also appear to maintain approximately same cell number.

Cell type	Doubling time
Iso B 11/17RS	29 hrs
Iso B 11/9'RS	35 hrs
A172 (01/22/07)	28 hrs

Table 2 Cell doubling times

These values are calculated based on the time points at which the cell count procedure was started on Day 3,6, and 9

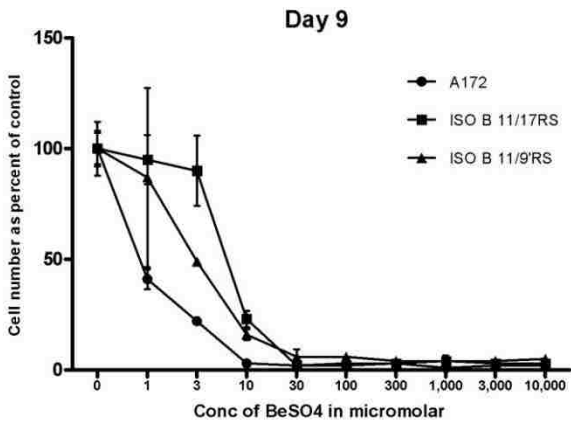
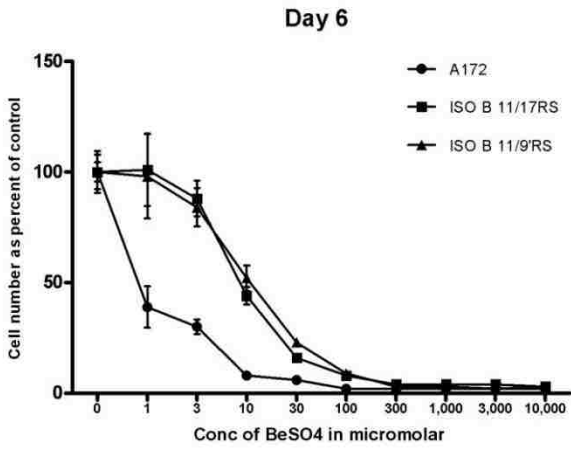
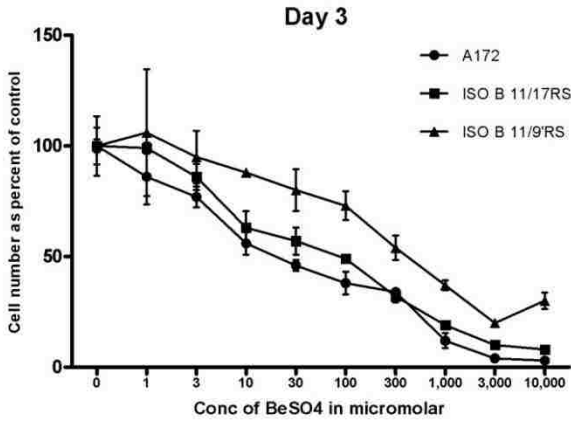


Figure 1 Iso cell types are resistant to beryllium compared to A172

Day 3,6 and 9 cell count data is plotted as percent of control cells (0µM of respective cell lines) shows that the Iso cell types of A172 cell line were able to adapt to beryllium and have higher resistance compared to regular A172. The data are presented as mean +/- Standard Deviation.

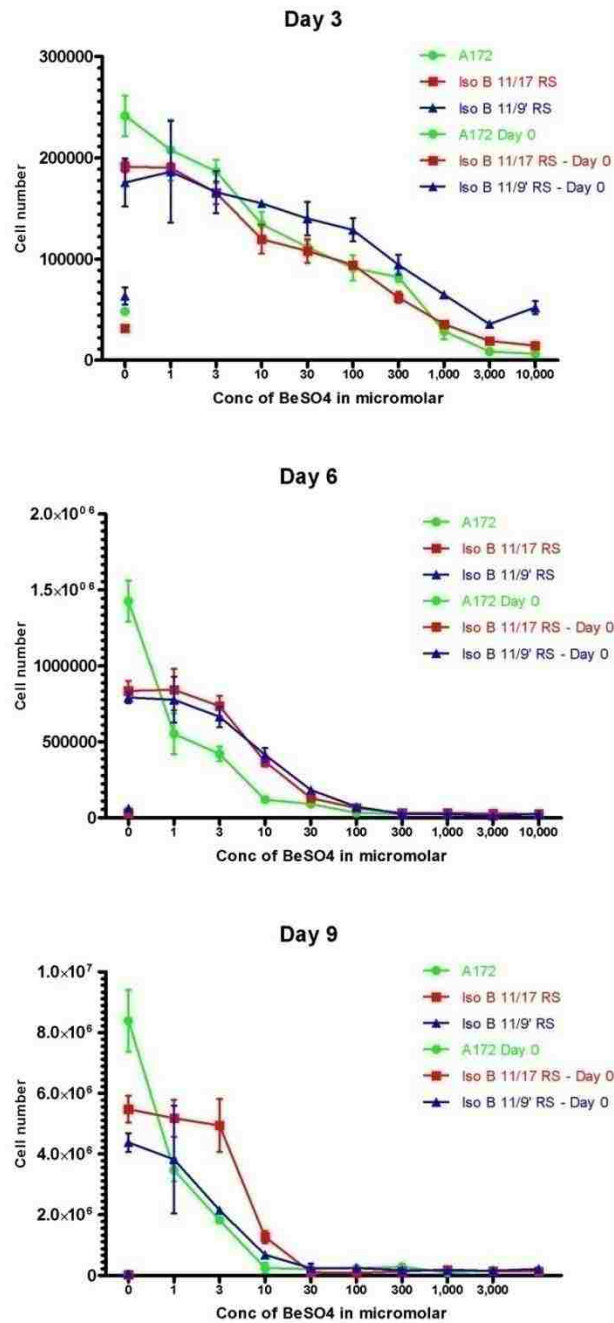


Figure 2 Cell doubling time of Iso cell type is greater than A172

The absolute cell counts with dilution factors of 8 for day 6 and 64 for day 9 incorporated into cell counts were plotted. Day 0 counts 48200, 31400 and 63466 for A172, IsoB 11/17 RS and IsoB 11/9' RS respectively. By end of day 3 it can be seen that A172 cell were replicating faster compared to the Iso cell types. Among the Iso cell types Iso B 11/17 RS were having lower cell doubling time. The data are presented as mean +/- Standard Deviation.

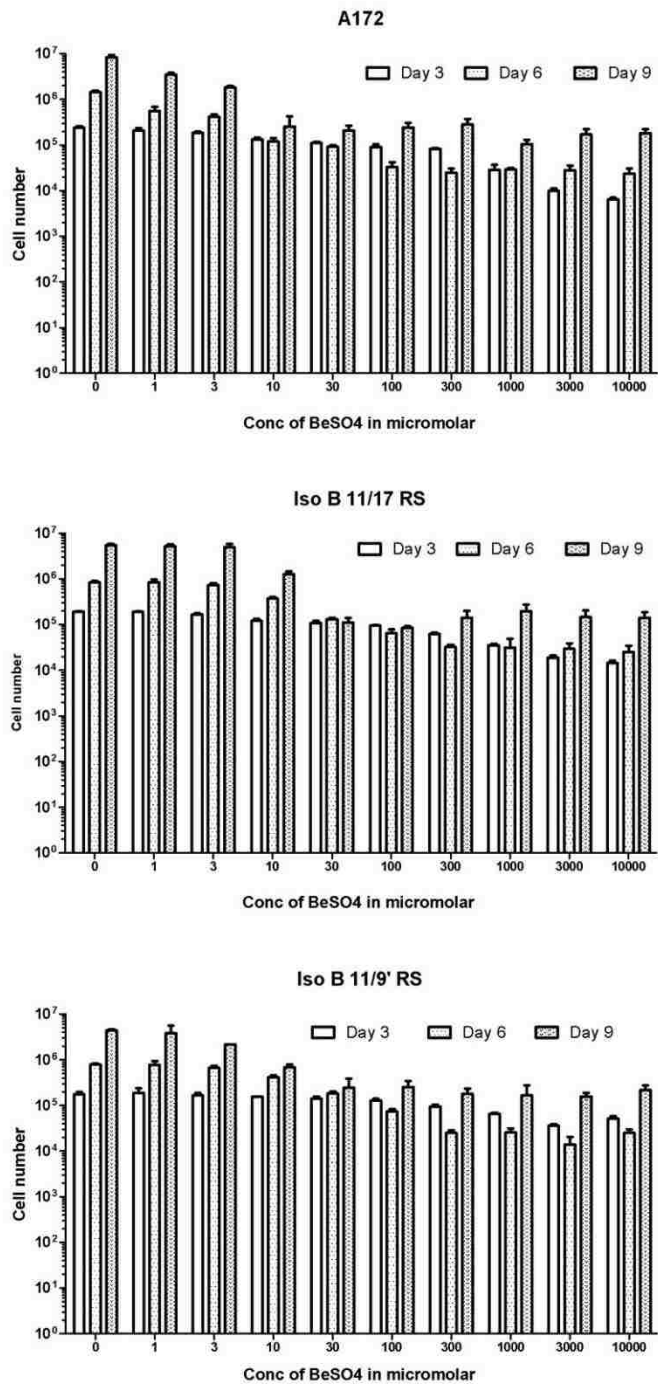


Figure 3 Cytostatic concentration of beryllium

Dilution factor included total cell numbers are plotted against beryllium concentration for Day 3,6 and 9. For A172 the minimum concentration of beryllium that can stop cells from dividing is 10μM. The cytostatic concentration for Iso cell types is 30μM. The data are presented as mean +/- Standard Deviation.

Appendix III Evaluation of p53 and p21 status and response to beryllium treatment in BV cell lines

BACKGROUND

Four different cell lines were obtained from Dr. Bert Vogelstein's lab. These are HCT116 cells (Human colon carcinoma cells), one of these being a p53 knock out and another is p21 knock out along with two respective controls.

EXPERIMENT 1

Objective

To check the sensitivity of all 4 cell lines to beryllium dose.

Method

The cells are exposed to 0, 1, 3, 10, 30, 100, 300, 1000, 3000, 10,000 μM doses of Be for a period of 6 days. The cell counts are taken at 3rd and 6th day. The experiment was set up in 60mm dishes. 75000 cells/plate at 3 mL per plate were seeded on day -1.

Cell count procedure

Old media is removed and cells are washed with P.B.S one time.

0.5 mL of trypsin EDTA is added to the cells and plate is incubated for 3 minutes at 37°C. 2.5 mL of RPMI is added to inactivate the trypsin and cells are resuspended in RPMI. 100 μL of cell suspension is transferred into a coulter counter cup filled with 9.9 mL of isotone and used for getting cell counts.

3rd day counts

7 mL of RPMI with 8/7 times the required concentration BeSO_4 for is prepared in the pre-labeled 15 mL tubes.

100 μL of cell suspension is used for cell counts. 1ml of the cell suspension is added to the respective 15ml tubes with BeSO_4 RPMI, giving 1:8 diluted cell suspension of which 3 ml will be seeded to new pre-labeled 60 mm dishes.

6th day counts

Old RPMI is removed and cells are washed with P.B.S one time. 0.5mL trypsin is used for 3 minutes at 37°C. Trypsin is inactivated and cells are suspended by using 2.5 mL RPMI. 100 µL of cell suspension is transferred into 9.9 mL of isotone for cell counting.

Result

Due to abnormal counts obtained at various concentrations for each cell line no conclusions could be drawn from the data (Fig 1).

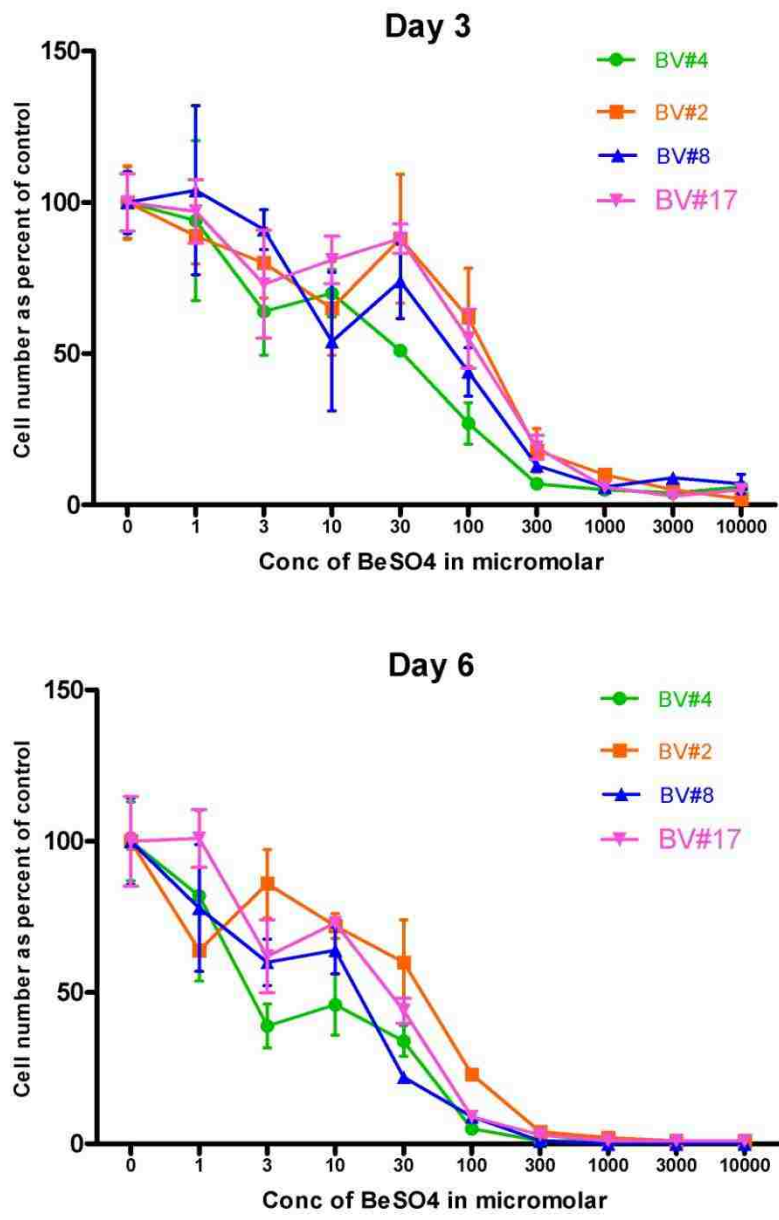


Figure 1 Cell count data for BV cells

Cells were trypsinized and resuspended in RPMI with beryllium concentration calculated to compensate dilutions. 100µL of cell suspension was mixed with 9.9mL isoton solution. Coulter counter was used to get cell counts. Due to large standard deviation in cell numbers and abnormal counts obtained the data could not be used to arrive at any useful conclusions. The data are presented as mean +/- Standard Deviation.

EXPERIMENT 2

Objective

To identify p53, p21 knock outs and controls.

Method

1. Western blot
2. Real time PCR

1. Western Blot

Cells for BV#2, #4, #17, #8 were setup in 100mm plates and harvested when the confluency reached 95% using RIPA extraction protocol. Extracted samples were mixed with 5x Lamelli sample buffer and boiled for 3 minutes. BCA assay was performed on remaining cell supernatant to determine total protein concentration.

PAGE was performed using 9% acrylamide gel. Primary antibody treatment given using p21 and p53 antibodies over night. Corresponding secondary antibody treatment done and blots were developed using ECL+ reagent. Typhoon was used to scan the blot.

Result

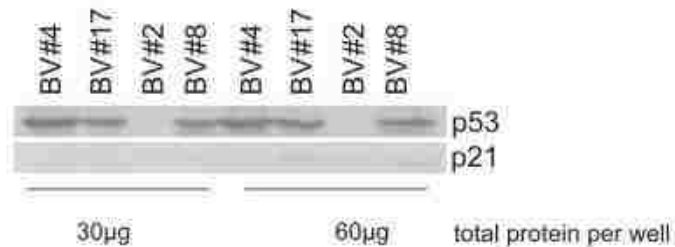


Figure 2: p53 and p21 status in BV cell types

BV#2 – p53 band absent - p53 knock out cell type; BV#4 – p21 band absent - p21 knock out cell type; Both BV#17 and BV#8 are expressing p53 and p21 protein and are control cell types.

2. Real time PCR

Cells for each cell type, BV#2, #4, #17, #8, were cultured in 100mm plates and RNA was extracted from the cells. Reverse transcription was done to generate cDNA. cDNA diluted and used in Real Time PCR reactions to screen for p21 gene expression along with Actin and GAPDH.

Result

Extremely high concentration of BeSO₄ at 1 mM was not able to induce p21 gene expression in p53 knocked down BV#2 cell type (Fig 3). At 100 μM BeSO₄ dose the control cell line BV#8 did not show any response in the form of p21 mRNA expression. The results suggest that BV cell lines i.e., HCT116 are resistant to beryllium dosing and p21 expression in these cell is p53 dependent in response to beryllium treatment.

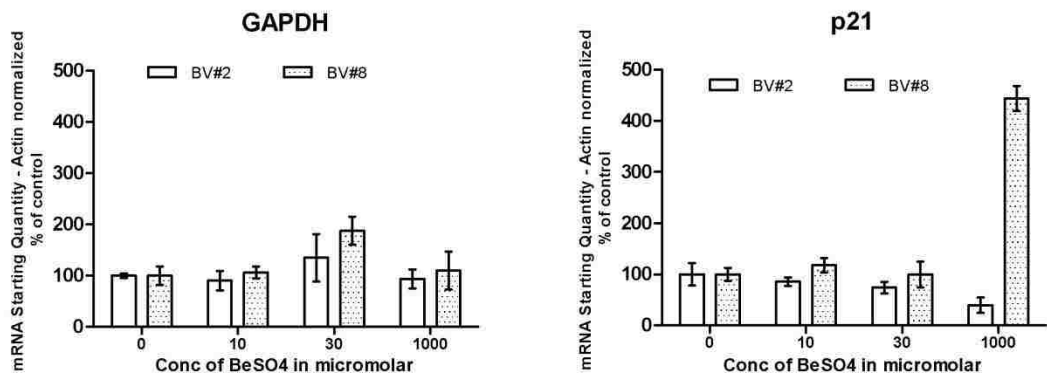


Figure 3 p21 gene expression in p53 knock down BV cell line

Cells were cultured in +/- Be RPMI for 48 hours at 0, 10, 30 and 1000 μM concentrations. The starting quantity (SQ) of mRNA was measured using real time PCR techniques. Values are normalized with Actin and expressed as percent of control. The data are presented as mean +/- Standard Deviation.

BV#2 cell line is p53 knock out variety and BV#8 is a control cell line. As seen from data the p21 gene is not expressed in BV#2 cell line even at very high concentration of beryllium dose of 1mM. BV#8, with functional p53, required a very high dose to induce a response in the form of p21 gene expression increase.

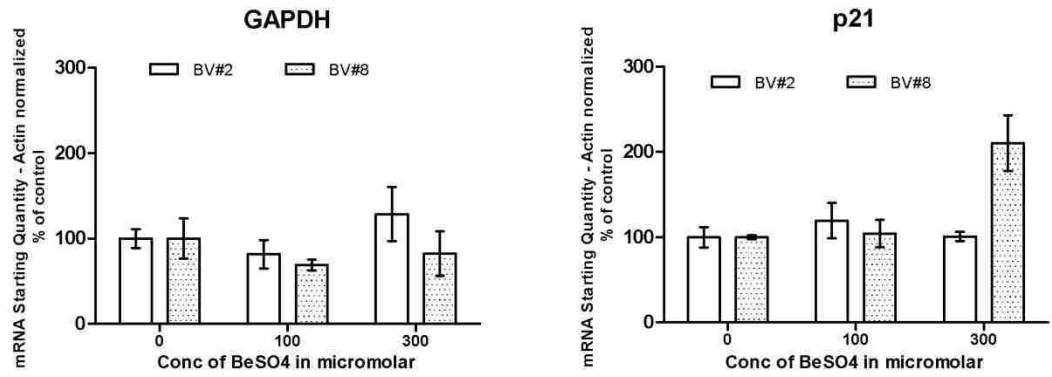


Figure 4 p21 gene expression in p53 knock down BV cell line

The experiment in Figure 3 is repeated with different concentrations of beryllium here. Exposure time is 48hours. The data shows that BV#8 ,the control cell line with wild type p53 gene, is able to resist effect of beryllium upto 100 μ M. The p53 gene expression at 300 μ M is doubled compared to control. On the other hand BV#2, the p53 knock out (p53KO) variety has no expression of p53 gene. The data are presented as mean +/- Standard Deviation.

Appendix IV Optimization of siRNA for p53 knock down and generating stable p53 knock down shRNA A172 clones

EXPERIMENT 1

BACKGROUND

TP53 validated stealth RNAi (Cat No. 45-1492) was purchased from Invitrogen. The kit contains two Validated Stealth RNAi Duplex oligos. Cells treated with duplex 1 and 2 are labeled as KO1 and KO2 respectively.

Objective

To test if the new TP53 validated stealth RNAi is able to induce efficient knock down of p53 expression.

Method

HFL-1 young cells at passage 16, hTERT HFL-1 and A172 cells were cultured in 60mm plates and treated with validated stealth RNAi duplex oligos according to kit instructions. The cells were then treated with beryllium and western blotting method was used to verify presence of p53 protein.

Result

BLOCK-iT fluorescent Oligo was used to get an assessment of the transfection efficiency. The fluorescein-labeled duplex localized in nucleus as expected (Fig 1).

Validated stealth kit was able to induce p53 knock down efficiently in the three different cell types tested. The knock down was maintained over a period of 48 hours (Fig 2).

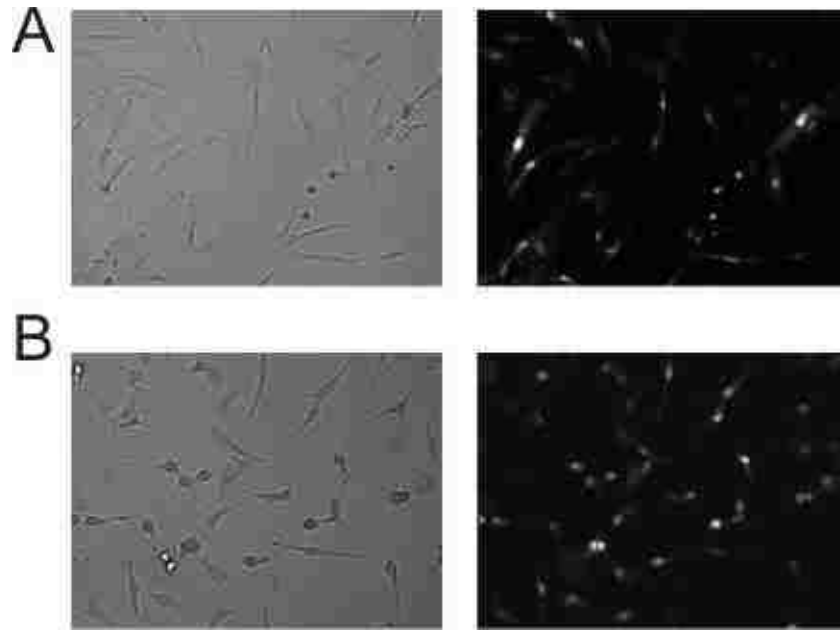


Figure 1 Nuclear localization of fluorescent tagged Oligos

Left panels show the phase contrast images of the fluorescent field shown on right. Fluorescent image is taken with FITC filter having 494nm excitation and 519nm emission filters.

hTERT HFL-1 (A) and A172 (B) were treated with BLOCK-iT and images taken at the end of 14 hours.

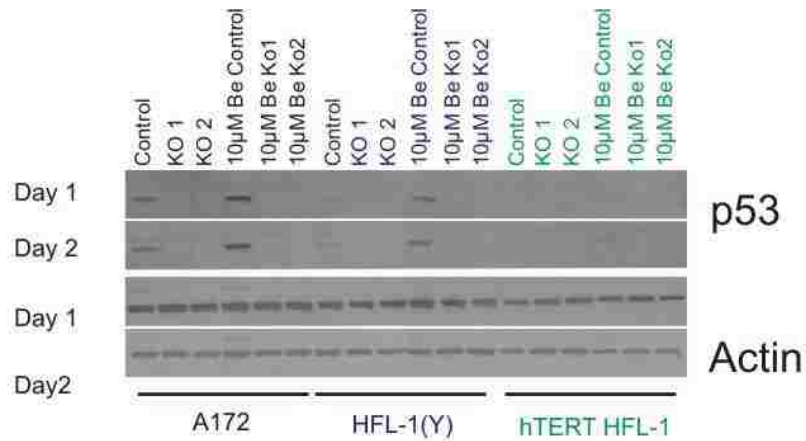


Figure 2 p53 protein screening in KO cells using western blot

A172, HFL-1 young and hTERT HFL-1 cells were treated with validated stealth RNAi to induce p53 knock down and then cultured for another 24hours (Day1) and 48hours (Day 2). The total protein concentration in HFL-1(Y) and hTERT HFL-1 samples was low so the samples used without normalization. But the Actin band shows that these samples were loaded at almost same total protein concentration. A172 samples were normalized using BCA assay.

EXPERIMENT 2

BACKGROUND

The TP53validate stealth RNAi kit was effective in inducing knock down of p53 in the different cell types tested. However, It was observed that cell morphology changed considerably after they receive Stealth RNAi treatment. According to the kit protocol the duplex sequences are required to be mixed with Lipofectamine 2000 and Optimum media. To improve the performance of kit by reducing damage to cells an experiment was designed to optimize the concentration of lipofectamine 2000 and duplex oligos.

Objective

To identify optimum concentration of duplex oligos and lipofecatmine 2000 that results in effective knockdown of p53 without causing changes in cell morphology.

Method

A172 cells were treated with the following combinations of lipofectamine and duplex oligos. 100% refers to kit recommended concentration.

100 % Oligo + 100% Lipofectamine 2000

50 % Oligo + 50% Lipofectamine 2000

100 % Oligo + 50% Lipofectamine 2000

Cells were then harvested using MPER at the end of 48, 72 and 96 hours. The samples were normalized using BCA assay and western blotting was done to check for p53 levels. As a control sample extract from A172 cells treated with beryllium was used.

Result

The combination #2 having 50% concentration of oligo as well as lipofectamine was effective as 100% concentration upto 3 days. The cell death and morphology changes were lower than 100% concentration. At 4 days both 100% and 50% dosed cells showed recovery of p53 activity. It was decided to use 50% concentration for all future experiments with repeat dosing at the end of 72 hours for experiments that require p53 knock down to be maintained for longer periods.

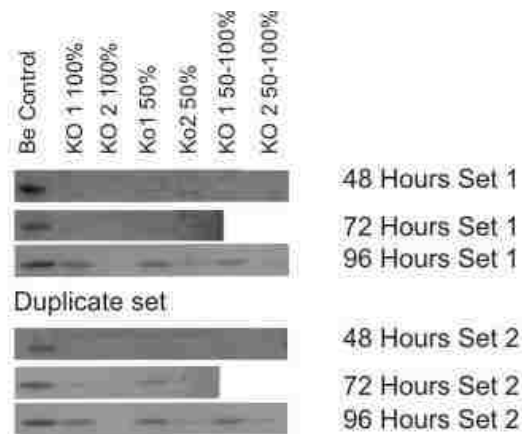


Figure 3 Optimization of Lipofectamine 2000 and Oligo concentrations

100% refers to A172 cells treated with Kit recommended concentration (combination 1), 50% lanes have cells treated with combination 2 at 50% of kit recommended concentrations, 50-100% lanes refer to samples from cells that were dosed with 50%

Lipofectamine 2000 and 100% Oligo, combination 3. Combination 3 samples were not run on gel due to limited number of wells on gel. Cells receiving 100% of lipofectamine 2000 showed severe morphology changes and higher cell death. MPER lysed samples are resolved on PAGE and probed with p53 (DO-1) antibody

EXPERIMENT 3

BACKGROUND

Integrating specific short hairpin RNA (shRNA) constructs into genomic DNA can induce targeted gene knock down. We have used p53 shRNA (h) Lentiviral Particles (sc-29435-V) along with Control shRNA Lentiviral Particles (sc-108080) as negative control, from santa cruz biotechnology, inc, to generate stably transfected A172, HFL-1 and hTERT HFL-1 cells.

Method

8000 cells of each cell type were suspended in 200 μ L of RPMI and into 96 well plate. The cells were allowed to attach overnight. The lentiviral particles were diluted in RPMI with polybrene (Sigma Cat No. 107689) to get MOI of 0.5, 1, 2,4, 6 and 8. After an overnight incubation regular viral RPMI was replaced with regular RPMI. Puromycin at 750ng/mL in RPMI was used to select and propagate successfully transfected cells. Cells were transferred to 60mm plates when wells in 96 well plate reached 90% confluency. Cloning cylinders (Sigma Cat No. C1059-1EA) were used to select single cell colonies for HFL-1 and A172 cells. The clones were further propagated and maintained under selection pressure using puromycin RPMI. hTERT HFL-1 cells used in this experiment were generated under puromycin selection. So the puromycin based selection in this experiment is not guaranteed to select for p53 knock down. So hTERT cells from high MOI wells of 96 well plate are saved.

The clones were verified for p53 knockdown using western blotting.

Result

The western experiment confirmed successful knock down of p53 protein in the clones selected. The transfected cells showed two bands for p53 with the lower band corresponding to the band seen in normal A172 sample. The lower molecular weight band is of lower intensity compared to control transfected cells. It has to be noted that single band was seen in other western blot experiments done with these A172 clones using a higher percentage gel.

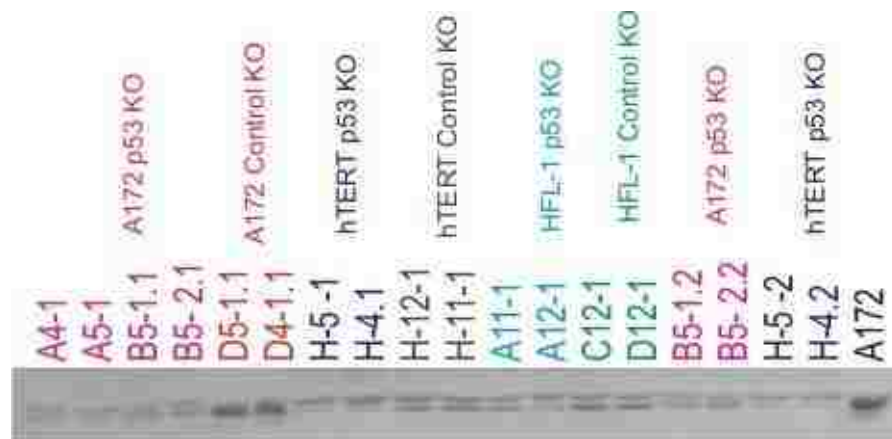


Figure 4 p53 protein screening of A172 p53 shRNA knock-down clones

Lentiviral treatment was able stably integrate shRNA to introduce p53 knockdown in the selected clones. hTERT HFL-1 cells were previously selected under puromycin and the cells may be a mixture of p53 knock down and p53 normal cells. A172 48 hour beryllium treated cell sample was used as a reference for p53 protein.

Appendix V Beryllium on the activity of GSK 3 beta

BACKGROUND

Work done by previous graduate student included study of the effect of beryllium on the activity of glycogen synthase kinase-3beta (GSK 3 β). The western blot results presented in this chapter are an extension of her work.

EXPERIMENT 1

Objective

To screen different cell lines for expression of GSK 3 β and phosphor-Glycogen Synthase (pGS).

Method

A172, S91, PC12, RKO, MCF7 and HFL-1 cells were treated with beryllium 30 and 100 μ M, lithium 20mM, 1-Azakenpallone - AKPL – 10 μ M and 5-Iodo-indirubin-3'-monoxime – INDI - 20 μ M. MPER with protease and phosphatase inhibitors was used to harvest the cells at the end of 48 hours.

Result

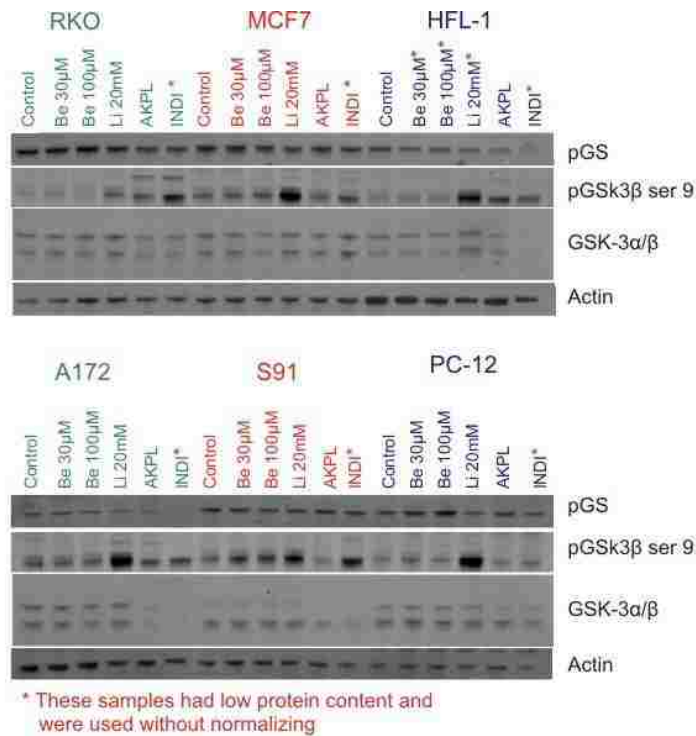


Figure 1 Expression of total GSK, pGSK3β and pGS in 6 different cell lines

Samples from all cell lines are not properly normalized. Both GSK-3α (~51 kDa) and GSK-3β (~47 kDa) proteins are seen in GSK-3 α/β panel. It appears that HFL-1 and S91 cell lines may be having different levels of the enzyme.

With respect to pGS and pGSK3β ser9 general observation is that both Synthetic inhibitors and Lithium were able to inhibit phosphorylation of GS compared to beryllium. Both Synthetic inhibitors and Lithium were able to induce phosphorylation of GSK3β at serine 9. The response of all cell lines to the different treatments is not same. Most of the HFL-1 samples and INDI samples were loaded to give maximum total protein load per well due to low protein concentration.

EXPERIMENT 2

Objective

To evaluate the influence of beryllium on the activity of GSK 3 β as compared to Lithium and synthetic inhibitors.

Method

Phosphorylation of Glycogen Synthase and Tau, the substrates of GSK 3 β , will be used as measure of increase or decrease of the GSK 3 β enzyme activity.

A172, HFL-1 Young cells at passage 19 and PC12 were treated with Beryllium - 10,30 and 100 μ M, Lithium – 10, 20, 30mM, 1-Azakenpaullone - AKPL – 10 μ M, Alsterpaullone - ALPL – 10 μ M. Cells were harvested using MPER with protease and phosphatase inhibitors at the end of 24 and 48 hours.

1-Azakenpaullone and Alsterpaullone are synthetic inhibitors of GSK 3 β (Leost et al., 2000).

The samples were probed for pGS, Tau, pTau and actin.

Result

Images obtained from western blotting were faint and not clean thus making band identification difficult. The images below are labeled for sample lanes but not for specific bands of the proteins.

The molecular weight marker shown for reference is for 4-12% gels as provided by manufacturer but the gels used for the experiment are 10% gels.

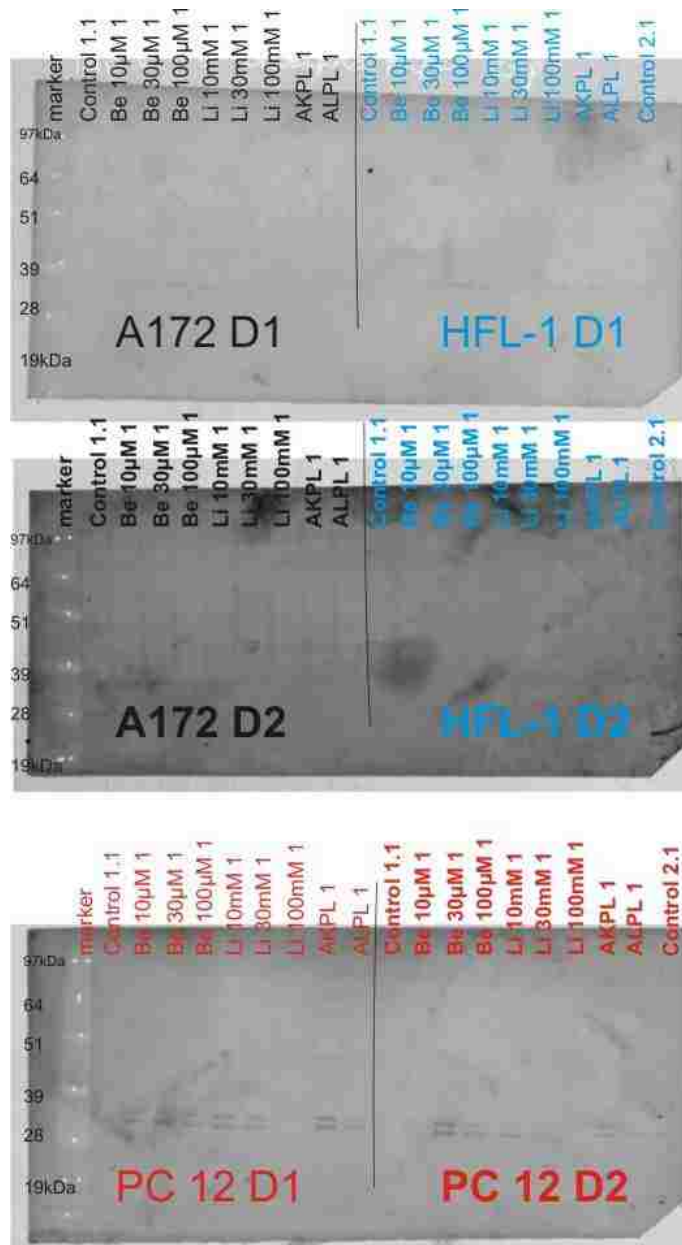


Figure 2 Tau(C-17) protein expression in A172, HFL-1 and PC12

A172, HFL-1 and PC12 cells were treated with beryllium, Lithium and synthetic inhibitors as described in methods. The MPER extract of these cells was normalized using BCA assay. Protein samples are resolved using PAGE and probed with Tau(C-17) antibody. A172 and HFL-1 blots have almost no bands. May be due to low protein conc combined with much lower levels of Tau in these cell lines. PC12 samples show some faint bands. The duplicate set of samples for PC12 were also run on gel and also pTau levels were probed in PC12 samples only (Figure 2). The expected molecular weight for Tau(C-17) is 46-80 kDa.

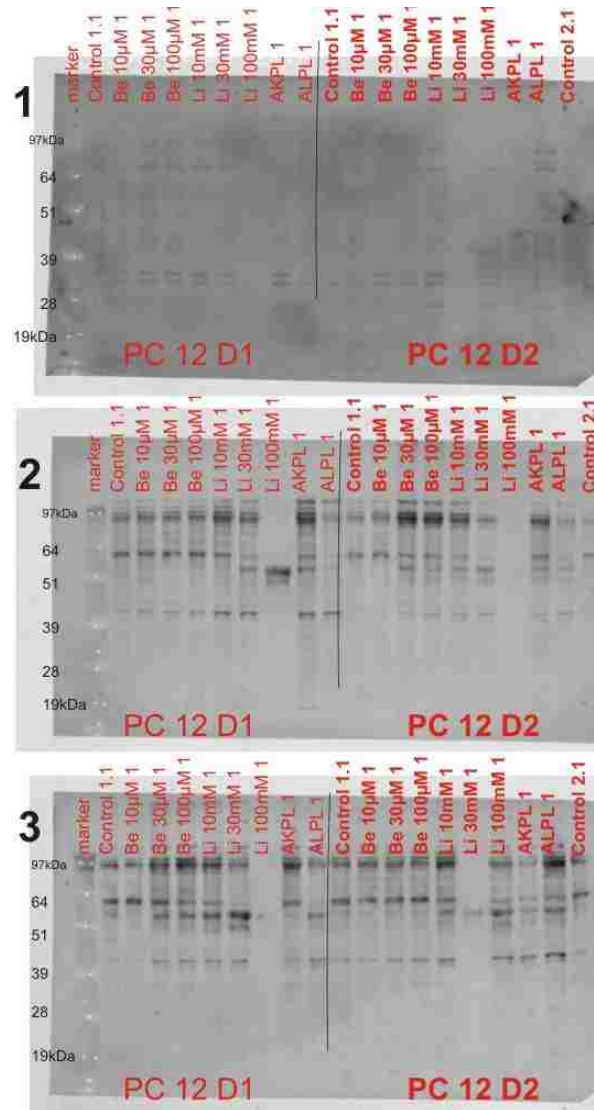


Figure 3 pTau (Ser 396) in PC12 cells

The duplicate set of samples from PC12 in figure 1 are probed with Tau(C-17) antibody. 2 and 3 are blots for PC12 replicate set of samples probed for pTau (Ser 396). The expected molecular weight for pTau (Ser 396) is 45-68 kDa.

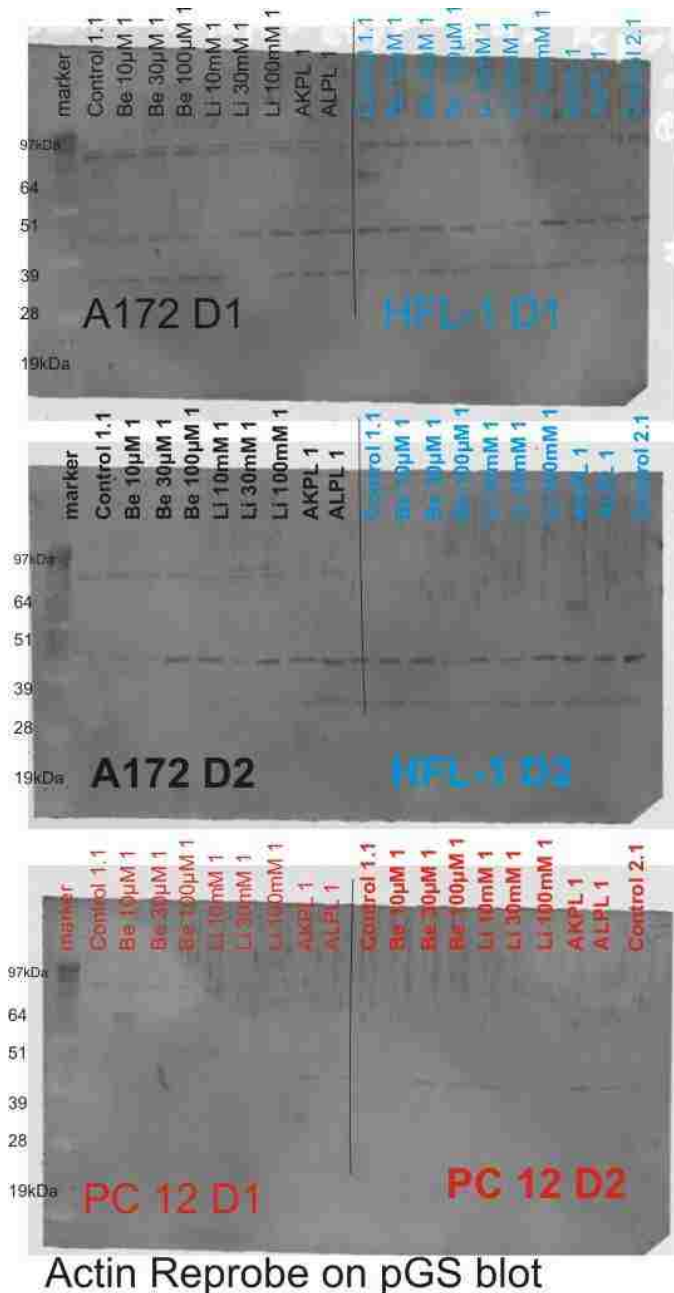


Figure 4 Actin levels to check total protein normalization

Actin reprobe on phospho GS blot shows that some of the samples have very low protein. It indicates inefficient transfer or problem with primary antibody treatment. Molecular weight of Actin is approximately 43 kDa

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Appendix VI TUNEL assay protocol

Reagent List

Equilibration buffer: Dilute 5x Terminal Transferase buffer.

TdT: 30U/uL from TERMINAL DEOXYNUCLEOTIDE 1500U Promega

Cat#M1875

Required at 30U per reaction according to protocol

PBS from HyClone Cat# ATH 32652

2x SSC: Dilute the 20x SSC solution left over from promega kit in DI water.

4% paraformaldehyde: prepared from paraformaldehyde solid (Sigma P6148).

Weigh 1.6g into 35mL P.B.S. Heat solution on hot plate set to 1 or 2. Use thermometer to monitor temperature at 65°C. Use magnetic stirrer to stir the solution. Cover the beaker with aluminum foil to avoid splatter. When the solution turns clear stop stirring and allow cooling. Carefully measure the volume of solution remaining in the beaker and make up volume to 40mL. Store in 50mL tube at 4°C. 4% paraformaldehyde is diluted to required concentration in P.B.S.

0.2% Triton X100: Sigma Cat# T9284 - Mix 80uL + 40mL P.B.S

70% ethanol: 95% Denatured Ethanol (Cat# BDH1158-4) is diluted to 70% by mixing 29.4mL of alcohol with 10.5mL of P.B.S.

DNase I : From Biolabs cat# M0303S. Stock concentration is 2000U/mL. Diluted in DNase I buffer to 1U/reaction

Nucleotide mix

	Required Conc	Stock Conc	
Fluorescein-12-dUTP	50uM	1mM	Fisher Scientific Cat No.: FERR0101
dATP	100uM	1mM	TQAC131- omega Bio-tek - VWR Secondary stock diluted to 1mM from 100mM stock in Tris-Hcl 10mM pH7.6
Tris-HCl pH 7.6	10mM	10mM	

TdT labeling mix

	Stock	Required for 1 Rxn
Equilibration buffer	5x	Terminal Transferase Buffer Promega Cat#M1893 Dilute to half with DI water to get 2.5x, use 100uL per reaction
Nucleotide mix	Use mix prepared above	10uL for each reaction
TdT enzyme	30U/uL	TERMINAL DEOXYNUCLEOTIDY 1500U Promega Cat#M1875 Use 1uL for each reaction

Procedure

Fixing cells

1. Cells grown in tissue culture plates are trypsinized by exposing cells to 0.05% trypsin for 3 minutes at 37°C followed by deactivating with used RPMI collected into a pre-labelled tubes before adding trypsin. The cell suspension was collected into a prelabelled 15mL tubes.
5. The cell suspension is centrifuged at 300xg, 4°C for 6minutes.
6. Supernatant is removed and cell pellet is gently dispersed by tapping the tube.
7. Cells are washed twice in P.B.S.
8. P.B.S is removed and tube is tapped gently to disperse the cells.

9. 1mL of 1% paraformaldehyde is added to the cells and incubate on ice for 20minutes.
10. Paraformaldehyde is removed by centrifuging cells at 300xg, 4°C for 5 minutes.
11. P.B.S wash is given to cells twice
12. After removing P.B.S, 70% cold ethanol is added to cells.
13. The cells are stored at 4°C till they are processed by TUNEL assay.

TUNEL assay

14. Remove ethanol from samples by centrifuging and wash the cells twice with P.B.S.
15. Start processing DNase samples first.
16. Equilibrate cells in equilibration buffer for 10minutes.
17. Remove buffer by centrifuging cells at 300xg, 4°C for 5 minutes.
18. Add DNase I enzyme and incubate at room temperature for 10 minutes.
19. Remove DNase I enzyme by centrifuging at 300xg, 4°C for 5 minutes and wash cells with P.B.S one time.
20. Prepare nucleotide mix and TdT labeling mix as required for the number of samples.
21. To all samples add equilibration buffer and leave at room temperature for 10minutes.
22. Centrifuge and remove excess buffer.
23. Add 100µL of TdT mix to each tube and incubate at 37°C for 1 hour 45 minutes.
24. Add 500µL of 10mM EDTA to stop the reaction.
25. Centrifuge at 300xg for 10 minutes.
26. Wash cells with P.B.S twice and remove excess P.B.S by centrifuging at 300xg for 10minutes.
27. Resuspend cells in 100µL P.B.S and transfer the cells into flowcytometry tube.

28. Save the tubes on ice and process using flowcytometer set to read FITC label.

** It was observed that FITC label can interfere with PI stain readings. It is preferable to prepare a parallel set of samples for DNA content analysis instead of using FITC cells to double label with PI stain.

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VITA

Graduate College
University of Nevada, Las Vegas

Priyatham Gorjala

Degrees:

Bachelor of Science, Bio-Physics, Genetics and Chemistry, 2001
Osmania University, India.

Master of Science, Medical Biochemistry, 2004
Manipal Academy of Higher Education, India.

Publications:

Gorjala P and Gary RK. (2010) Beryllium sulfate induces p21 CDKN1A expression and a senescence-like cell cycle arrest in susceptible cancer cell types *Biometals* 23:1061-1073.

Dissertation Title:

Characterization of beryllium as a novel agent to study cell cycle arrest and cellular senescence

Dissertation Examination Committee:

Chairperson, Ronald K. Gary Ph.D
Committee Member, Bryan L. Spangelo Ph.D
Committee Member, Ernesto Abel-Santos Ph.D
Committee Member, Dong-Chan Lee Ph.D
Graduate Faculty Representative, Frank van Breukelen Ph.D