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Beryllium is a potent and unique GSK-3β inhibitor with potential to differentially regulate glycogen synthase and β-catenin

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BERYLLIUM IS A POTENT AND UNIQUE GSK-3β INHIBITOR WITH POTENTIAL TO DIFFERENTIALLY REGULATE GLYCOGEN SYNTHASE AND β-CATENIN

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ABSTRACT

Beryllium is a potent and unique GSK-3β inhibitor with potential to differentially regulate glycogen synthase and β-catenin

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Glycogen Synthase Kinase-3β (GSK-3β) is an important serine/threonine kinase that phosphorylates/regulates diverse and metabolically important proteins. Some of the important substrates of GSK-3β are glycogen synthase, tau, β-catenin, cyclin D1, axin, c-jun, c-myc, Heat Shock Factor–1, BCL-3, CREB, Histone H1.5, mdm2, p21 (CIP1), pyruvate dehydrogenase and many more. De-regulation of GSK-3β has been implicated in diseases like cancer, Alzheimer's disease, bipolar disorder and type 2 diabetes mellitus. Currently, GSK-3β is one of the most widely studied proteins and there is a great interest in developing potent and efficient GSK-3β-inhibitors for research as well as therapeutic purposes. We demonstrate that beryllium in the form of $BeSO_4$ salt is a much more potent GSK-3β-inhibitor compared to LiCl.

We observed that one of the unique properties of beryllium is its modus operandi to regulate the inhibitory Ser-9 phosphorylation of GSK-3β in a cell type specific manner. Our study for the first time validates the potential of beryllium to function as a biologically relevant GSK-3β-

inhibitor. Beryllium induces a decrease in the phosphorylation of glycogen synthase in cultured NIH/3T3 cells. This decrease in phosphorylated form of glycogen synthase demonstrates the ability of beryllium to inhibit GSK-3β's kinase activity in treated cells. One of the most important substrates in relation to GSK-3β's inhibition is β-catenin – one of the downstream effector molecules of the Wnt signaling pathway. Our results in NIH/3T3 and A172 cells indicate that beryllium has minimal effect on Wnt signaling/ β-catenin pathway compared to other established GSK-3β-inhibitors. The minimal effect of beryllium at physiologically effective concentrations on Wnt signaling/β-catenin pathway indicates that it could be a more pathway specific inhibitor. One of the most intriguing discoveries of our study has been the lithium induced increase in the phosphorylated form of β-catenin, which is against the "accepted dogma". Here we propose an alternative model explaining the regulation of the Wnt/β-catenin signaling pathway by lithium.

Use of GSK-3β-inhibitors for therapeutic purposes presents the risk of inducing cancer in patients due to accumulation of β-catenin, an oncogene activator. The unique ability of beryllium to repress the kinase activity of GSK-3β without inducing a heavy nuclear localization of β-catenin might provide an opportunity to develop a potentially potent, efficient, pathway-specific and biologically active GSK-3β inhibitor with minimal adverse effects.

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CHAPTER 1

OVERVIEW

Research purpose

GSK-3β is an important Ser/Thr kinase, which is involved in various physiologically important cellular pathways (Frame and Cohen, 2001; Doble and Woodgett, 2003; Grimes and Jope, 2001). De-regulation of GSK-3β is directly linked to the development of different diseases such as type 2 diabetes milletus, Alzhemier's disease and cancer (Grimes and Jope, 2001; Henriksen and Dokken, 2006; Smalley and Dale, 1999; Peifer and Polakis, 2000). There is great deal of interest in understanding the precise role of GSK-3β in the signaling pathways. Inhibitors act as an irreplacable tool to examine the role of an enzyme/protein in different signaling networks. Different categories of GSK-3β inhibitors are available and the most important among them is the small metal cations group. Lithium is a monovalent cation and is the most well characterized metal cation inhibitor of GSK-3β with an IC_{50} value of 12 mM (Ryves et al., 2002; Mudireddy et al., 2014). Li^{+} is a fairly specific GSK-3β inhibitor but is physiologically effective only at millimolar concentrations. Be^{2+} is a divalent cation capable of inhibiting GSK-3β (Ryves et al., 2002). We have used beryllium in the form of $BeSO_4.4H_2O$ and documented some of the different outcomes it induces in mammalian cell cultures in its role as a GSK-3β inhibitor. Emphasis was laid to understand and validate the effect of beryllium on GSK-3β-substrates or target proteins.

The present study can be summarized as follows:

- Chapter 1 – Overview and research questions.
- Chapter 2 - A brief introduction and information available about GSK-3β.

- Chapter 3 - We have analyzed the effect of beryllium salt on the different mammalian cell lines. This information was used in selecting cell lines that are most suitable for studies involving beryllium. Cell lines that are resistant or too sensitive to beryllium salt were not used for further studies.
- Chapter 4 - The effect of $BeSO_4$ on the kinase activity of GSK-3β was analyzed and Be^{2+} was found to be a more effective GSK-3β inhibitor compared to Li^{+}. Emphasis was laid on understanding the effect of beryllium on the Ser-9 phosphorylation status of GSK-3β. It is also observed that unlike lithium, beryllium cannot induce a clear increase in the Ser-9 phosphorylation of GSK-3β in certain cell lines.
- Chapter 5 - In this study we demonstrate the intra cellular effects of beryllium on GSK-3β substrates like glycogen synthase and β-catenin for the first time ever in cell culture. This study will be helpful in validating the ability of Be^{2+} to function as a biologically active GSK-3β inhibitor.
- Chapter 6 - We have used RT-PCR to analyze the effect of Be^{2+} treatment on the expression of certain genes. Genes which seems to be responding to beryllium were selected for this study depending on the microarray data available in our lab.
- Chapter 7 – Research summary and future directions.

Research questions

Beryllium is a known GSK-3β inhibitor, which competes for the Mg^{2+} and ATP binding sites of GSK-3β (Ryves et al., 2002). There is a great dearth of information regarding the role of beryllium as a GSK-3β inhibitor. This study is an attempt to establish whether Be^{2+} is an efficient GSK-3β inhibitor. Following are few important research questions we tried to address through this study.

1. Is Be^{2+} a potent GSK-3β inhibitor and how well is it tolerated by mammalian cell lines?

Test: Inhibitory effect of Be^{2+} on the in vitro kinase activity of pure recombinant GSK-3β will be analyzed using a FRET based kinase assay. The cyto toxicity of Be^{2+} can be examined using a live/dead cell protease assay.

2. Is Be^{2+} a specific GSK-3β inhibitor?

    - Test: The effect of Be^{2+} on pure recombinant protein kinase A (PKA - a kinase involved in insulin signaling pathway) can be analyzed using a FRET based kinase assay. PKA phosphorylates GSK-3β (Fang et al., 2000; Li et al., 2000; Tanji et al., 2002) suggesting that PKA and GSK-3β work closely to one another. Hence PKA is a suitable candidate to analyze the specificity of beryllium towards other related kinases.

3. What is the effect of Be^{2+} on the Ser-9 phosphorylation of GSK-3β?

Test: Increase in the phosphorylation of Ser-9 residue on GSK-3β is closely associated with its regulation. The impact of Be^{2+} on the Ser-9 phosphorylation of GSK-3β will be investigated using western blotting. The effect of Be^{2+} on the Ser-9 phosphorylation of GSK-3β can be quantified by using flow cytometry and TR-FRET based methods.

4. What is the effect of Be^{2+} treatment on the proteins downstream of GSK-3β (if Be^{2+} is a potent GSK-3β inhibitor)?

Test: GSK-3β is an important Ser/Thr kinase, which phosphorylates its substrates and plays an important role in their regulation. If Be^{2+} is a potent GSK-3β inhibitor in treated cells – then its inhibitory effects can be validated by analyzing the phosphorylation status of the GSK-3β–substrates. The phosphorylation status of important GSK-3β-substrates like glycogen synthase, β-catenin or tau will be investigated via western blotting.

5. Can Be^{2+} regulate the Wnt signaling pathway like Li^{+}?

Test: GSK-3β is an important constituent of the Wnt signaling pathway. Inhibition of GSK-3β can cause nuclear localization of β-catenin, which in turn leads to activation of some oncogenes. It is necessary to investigate the effect of a GSK-3β inhibitor on Wnt signaling pathway. The role of Be^{2+} in regulating the Wnt signaling pathway will be investigated by studying the nuclear localization pattern of β-catenin via immunofluorescence, flow analysis and western blotting.

CHAPTER 2

GSK-3β: An important Ser/Thr kinase

Glycogen synthase kinase 3 (GSK-3) is an important serine/threonine kinase found in all eukaryotes. GSK-3 was first isolated in its homogenous form from rabbit skeletal muscle cells (Embi et al., 1980). The name glycogen synthase kinase refers to its ability to phosphorylate glycogen synthase, a key regulatory element of glycogen synthesis and insulin signaling pathway.

GSK-3 family

There are two isoforms of mammalian GSK-3: GSK-3α and GSK-3β encoded by distinct genes (Woodgett, 1990). GSK-3α is 51 kDa and GSK-3β is 47 kDa, the difference in the size of the isoforms is due to the presence of a glycine rich extension on the N-terminus of GSK-3α. The two isoforms of GSK-3 share only 36% identity in their last 76 residues on the C-terminal but their kinase domains are highly identical (98%) (Woodgett, 1990). The GSK-3 homologues are found in almost all eukaryotes and shares highly similar kinase domains (Ali et al., 2001). In spite of their identical kinase domains GSK-3α and GSK-3β are not functionally identical. The deletion of exon 2 of GSK-3β causes the death of mouse embryos at day 16 due to the extreme hepatocyte apoptosis induced liver degeneration. Functional GSK-3α cannot alleviate the lethality induced in mouse embryos due to homozygous deletions of exon 2 of GSK-3β (Hoeflich et al., 2000). The lethality observed in GSK-3β null mouse embryos cannot be alleviated by a functional GSK-3α indicating the importance of the protein/enzyme GSK-3β. However animal models representing GSK-3α knock out are viable but display enhanced insulin sensitivity (MacAulay et al., 2007).

Table 1a Important events in the field of GSK-3 research from 1963 to 1993

(Adapted from Cohen and Frame, 2001**) –** Reprinted by permission from Macmillan Publishers Ltd: [nature reviews molecular cell biology] **(**Cohen P, Frame S. 2001). The renaissance of GSK3. *Nature reviews Molecular cell biology* **2**: 769-776), copyright (2001)**)**

Year	**Research related GSK-3**	**Refrences**
1963	**Glycogen synthase was found to exist in active and inactive forms**	**Friedman and Larner, 1963.**
1964	**Insulin promotes the conversion of glycogen synthase to its active form**	**Craig and Larner, 1964.**
1978	**Insulin could inhibit the activity of GSK-3**	**Cohen, 1979.**
1980	**GSK-3 purified**	**Embi et al., 1980.**
1983	**Insulin induced dephosphorylation of glycogen synthase occurs at serine residues, which are targets of GSK-3**	**Parker et al., 1983.**
1986	**Li^+ treatment caused duplication of dorsal axis in *Xenopus* oocytes**	**Kao et al., 1986.**
1987	**A "priming phosphate" is required for GSK-3 to recognize its substrates**	**Fiol et al., 1987.**
1990	**Two isoforms of GSK-3: GSK-3 α and β idetified**	**Woodgett, 1990.**
1991	**GSK-3 phosphorylates Jun family proteins in vitro and negatively regulates them**	**Boyle et al., 1991.**
1992	• **Insulin inactivates GSK-3** • **GSK-3 phosphorylates Tau** • **Wingless (WNT homologue) found to inactivate GSK-3**	**Hughes et al., 1992.** **Hanger et al., 1992.** **Siegfried at al., 1992.**
1993	**eIF2B identified as a GSK-3 substrate**	**Welsh et al., 1993**

Table 1b: Important events in the field of GSK-3 research from 1994 t0 2014

1994	• **Protein kinases activated by mitogen leads to inactivation of GSK-3α** • **PI3K inhibition found to hamper GSK-3 inhibition**	**Sutherland and Cohen, 1994.** **Cross et al., 1994.**
1995	• **Insulin induced inactivation of GSK-3β through the Ser-9 phosphorylation** • **PKB/AKT phosphorylates GSK-3 in vitro at appropriate sites**	**Cross et al., 1995.**
1996	• **Li^+ ions inhibit GSK-3** • **GSK-3 regulates sub cellular distribution of β-catenin**	**Klein and Melton, 1996.** **Stambolic et al., 1996.** **Rubinfeld et al., 1996.**
1998	**Axin shown to exist in a complex with GSK-3β and β-catenin and promotes GSK-3β dependent phosphorylation of β-catenin**	**Ikeda et al., 1998.**
1999	**FRATtide inhibits GSK-3 mediated phosphorylation of axin and β-catenin, but not glycogen synthase.**	**Thomas et al., 1999.**
2000	• **Disruption of GSK-3β is lethal to mouse embryos** • **Specific small molecule inhibitors of GSK-3β developed** • **Insulin and Wnt regulates GSK-3β differentially**	**Hoeflich et al., 2000.** **Coghlan et al., 2000.** **Ding et al., 2000.**
2001	• **GSK-3 inhibitors shown to hamper neuronal apoptosis** • **Crystal structure of GSK-3 solved** • **Li^+ competes for the Mg^{2+} binding sites of GSK-3β**	**Cross et al., 2001.** **Dajani et al., 2001.** **Ryves and Harwood, 2001.**
2002	**Be^{2+} is a GSK-3β inhibitor and could be competing for Mg^{2+} and ATP binding sites of GSK-3β**	**Ryves et al., 2002.**
2003	• **GSK-3β interacts and positively regulates p53 activity** • **GSK-3β-TIMAP-PP1 feedback loop plays a critical role in the regulation of GSK-3β**	**Watcharasit et al., 2003.** **Zhang et al., 2003.**
2007	**Phosphorylation of TIMAP by GSK-3β activates PP1**	**Li et al., 2007**
2014	**Be^{2+} is a 1000 times more potent GSK-3β inhibitor compared to Li^+**	**Mudireddy et al., 2014.**

GSK-3β – A brief introduction

GSK-3β is a fascinating enzyme playing a central role in extremely diverse intra cellular signaling pathways like Wnt signaling and hedgehog pathway. GSK-3β regulates glycogen synthesis, gene transcription, apoptosis, protein synthesis and cellular differentiation in various cell types (Frame and Cohen, 2001; Doble and Woodgett, 2003; Grimes and Jope, 2001). The moniker glycogen synthase kinase doesn't justify the ability/range of GSK-3β to phosphorylate various metabolically and structurally important proteins. There is overwhelming evidence which establishes a plethora of different proteins as validated substrates of GSK-3β and they are tau, β-catenin, Cyclin D1, Axin, c-jun, c-myc, Heat Shock Factor–1, BCL-3, CREB, Histone H1.5, mdm2, p21(CIP1), pyruvate dehydrogenase and many more (reviewed in Sutherland, 2011). Phylogenetically GSK-3β is closely related to the cyclin dependent protein kinases, however a major difference is the preference of GSK-3β for primed substrates. The GSK-3β substrates have to be primed at $n + 4$ position with a phosphate moiety in order to be recognized by the enzyme (*n* is the site of phosphorylation by GSK-3β) (Fiol et al., 1987). The common consensus sequence that serves as the phosphorylation site of GSK-3β is Ser/Thr-Xaa-Xaa-Xaa-pSer/pThr (where pSer/pThr are the primed residues, Xaa – any amino acid and Ser/Thr is the target site of GSK-3β) (Fiol et al., 1987).

Table 2a. Proposed GSK-3β substrates

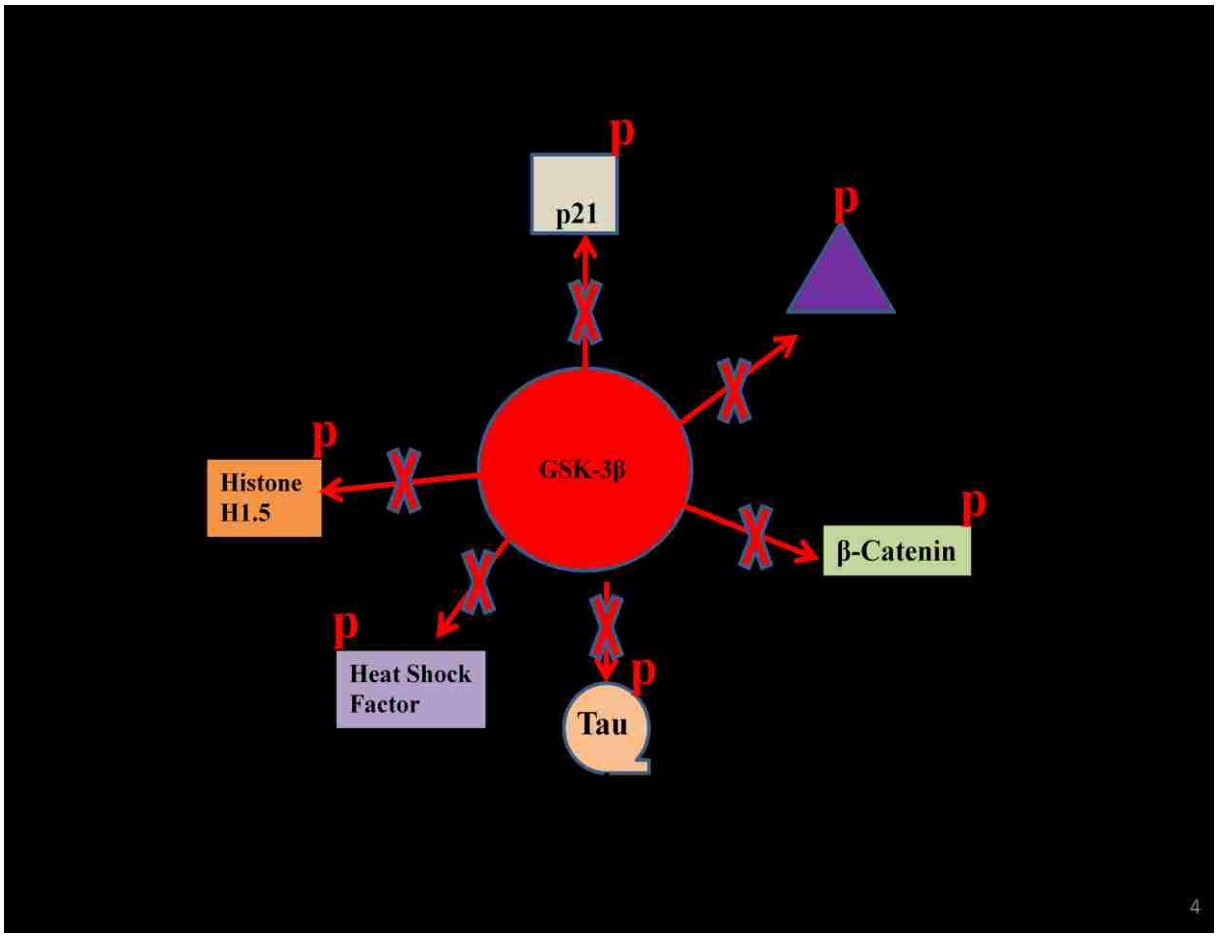
(Adapted from - What are the bonafide GSK-3 substrates, Sutherland, 2011 ; open access journal, International journal of Alzheimer's disease, 2011, 505607**)**

Proposed substrate	**Target residue(s)**	**Priming residue(s) and kinase**	**Effect of GSK-3β induced phosphorylation**	**Refrences**
Adenomatous Polyposis Coli (APC)	1501 1503	1505 (CK1) 1507 (CK1)	Regulates degradation	Ferrarese et al, 2007. Ikeda et al, 2000.
Axin	Ser-322 Ser-326 (putative)	Ser-330	Regulates stability	Ikeda et al, 1998. Yamamoto et al, 1999.
BCL-3	Ser-394	Ser-398 (ERK putative)	Regulates degradation	Viatour et al, 2004.
β-catenin	Ser-33 Ser-37 Thr-41	Ser-45 (CK1)	Regulates degradation	Ikeda et al, 1998.
Cyclin D1	Thr-286	NONE	Nuclear transport and degradation	Diehl et al, 1998.
Glycogen Synthase	Ser-640 Ser-644 Ser-652	Ser-658 (CKII)	Reduces activity	Rylatt et al, 1980. Parker et al, 1983.
Heat shock factor 1	Ser-303	Ser-307 (MAPK)	Reduces DNA binding	Chu et al, 1996.
Histone HI.5	Thr-10	NONE	Coincides with chromosome condensation	Happel et al, 2009.
c-jun, Jun B, Jun D	The-239	Thr-243	Reduces DNA binding	Boyle et al, 1991. Nikolakaki et al, 1993.

Table 2b. Proposed GSK-3β substrates

Proposed substrate	Target residue(s)	Priming residue(s) and kinase	Effect of GSK-3β induced phosphorylation	Refrences
mdm2	Ser-240 Ser-254	Ser-244 Ser-258 (CKI)	Promotes activity towards p53, reduces activity p53 levels.	Kulikov et al, 2005
c-myc, L-myc	Thr-58 Thr-62 (c-myc)	Ser-62 (ERK1/2)	Promotes degradation	Sears et al, 2000. Henriksson et al, 1993. Saksela et al, 1992.
p53	Ser-33(GSK-3β only)	Ser-37 (DNA-PK)	Increases transcriptional activity	Turenne and Price, 2001.
p21 CIP1	Thr-57	ND	Induces degradation	Rossig et al, 2002.
Protein phosphatase 1 G-submit	Ser-38 Ser-42 (human)	Ser-46 (PKA or p90RSK)	Not clear	Dent et al, 1989.
Protein phosphatase inhibitor 2	Thr-72	Ser-86 (CKII)	Inhibits inhibitor thereby activating PP1	Soutar et al, 2010. Aitken et al, 1984 DePaoli-Roach, 1984.
Tau	Ser-208 Thr-231 Thr-235 Ser-396	Thr-212 (DYRK)	Some phosphorylation sites regulate microtubule binding	Woods et al, 2001. Hanger et al, 1992. Yang et al, 1993.

Schematic diagram 1: De-regulation of GSK-3β activity towards its substartes has serious consequences



GSK-3β and the Wnt signaling

Wnts are a family of cysteine-rich, glycosylated, secreted protein ligands found in a wide variety of organisms ranging from hydra to humans (Miller, 2002). One of the pathways regulated by Wnt ligands is known as the canonical Wnt pathway or the Wnt/β-catenin pathway (Polakis, 2000; Seidensticker and Behrens, 2000; Sharpe et al., 2001). The Wnt signaling pathway is important for embryonic development, homeostatis, and development of central nervous system. (McMohan and Bradley, 1990; Patapoutian and Reichardt, 2000; Woodarz and Nusse, 1998; Moon et al., 1997; Polakis, 2000). GSK-3β is an important constituent of the Wnt signaling pathway and also the highly homologous wingless signaling pathway of drosophila. GSK-3β forms the β-catenin destruction complex along with APC (Adenomatous Polyposis Coli), axin, casein kinase 1 (CK1), protein phosphatase 2A (PP2A) and E3-ubiquitin ligase β-TrCP (Zeng et al., 1997; Behrens et al., 1998; Hart et al., 1998, Ikeda et al., 1998;Itoh et al., 1998; Salic et al., 2000; Kikuchi, 1999). β-catenin is an important substrate of GSK-3β and the GSK-3β mediated phosphorylation on the N-terminal region of β-catenin (Pefifer et al., 1994; Yost et al., 1996) marks it for ubiquitin-mediated proteosomal degradation (Hart et al., 1998, 1999; Behrens et al., 1998; Oxford et al., 1997; Winston et al., 1999; Kitagawa et al., 1999; Latres et al., 1999; Liu et al., 1999). β-catenin is one of the most important downstream effectors' of the Wnt signaling pathway.

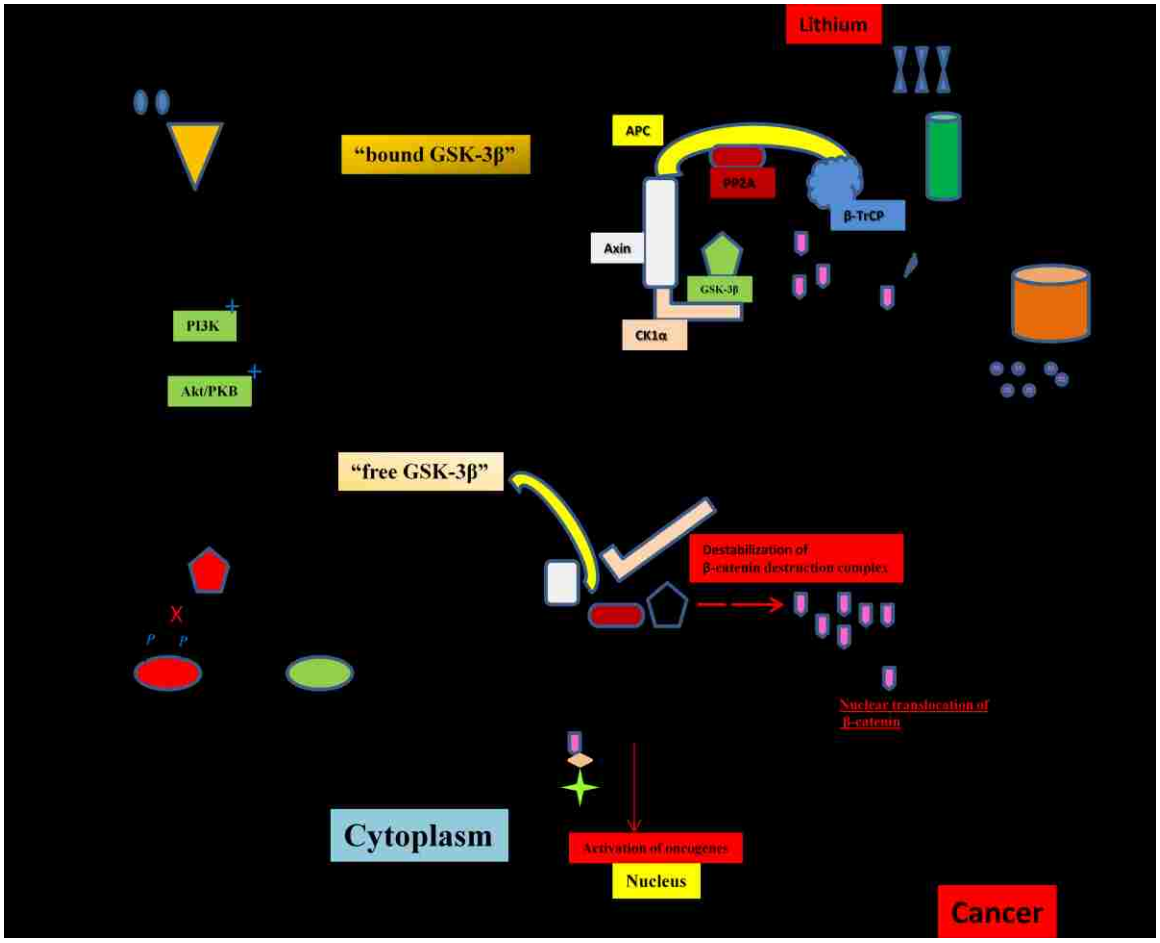
In the absence of Wnt ligands, the active GSK-3β in concert with axin and adenomatous polyposis coli (APC) phosphorylates β-catenin targeting it for ubiquitinylation and proteasomal degradation (Hart et al., 1998; Peifer and Polakis, 2000; Oxford et al., 1997; Aberle et al., 1997)

Wnt ligands bind to the extracellular domain of their receptor called frizzled protein thereby activating the Wnt signaling pathway. The activation of Wnt signaling cascade destabilizes axin which has a detrimental effect on the β-catenin destruction complex (Willert et al., 1999;

reviewed in Kikuchi, 1999). Wnt activation also blocks the phosphorylation of β-catenin by GSK-3β (Bilic et al., 2007; MacDonald et al., 2009; Metcalfe and Bienz, 2011) Thus Wnt activation inhibits GSK-3β phosphorylation of β-catenin and causes accumulation of β-catenin. These events ultimately lead to stabilization of β-catenin (Cook et al., 1996; Willert et al., 1999) and the accumulated β-catenin is translocated into the nucleus where it interactes with the TCF/LEF (T-cell factor/lymphocyte enhancer factor) proteins to form a protein complex (Huber et al., 1996; Molenar et al., 1996; Behrens et al., 1996). The β-catenin-Tcf/Lef protein complex binds to DNA and regulates the transcription of TCF/LEF target genes (Behrens et al., 1996; Dale, 1998; Brantjes et al., 2002). It is important to note that some of the target genes of β-catenin are proto oncogenes (Dale, 1998; Brantjes et al., 2002).

The relationship between GSK-3β and Wnt signaling pathway can be summarized as follows - a stable β-catenin destruction complex is required for the GSK-3β enzyme to phosphorylate β-catenin and it happens in the absence of Wnt signaling/ligands. Inhibition of GSK-3β by Wnt ligands prevents the phosphorylation and proteasomal degradation of β-catenin.

Schematic diagram explaining the role of GSK-3β in insulin pathway and Wnt/β-catenin pathway



Schematic diagram 2: Dissecting the role of GSK-3β in insulin and Wnt pathway. Lithium induced destability of the β-catenin destruction comeplex could be the possible reason for the nuclear localization of β-catenin in Li^+ treated cells. Whether beryllium can destabilize the β-catenin destruction comeplex or not is worth investigating further. It can be summarized that probably GSK-3β occurs in two cellular forms "free GSK-3β" and "bound GSK-3β" and beryllium seems to be mostly targeting the "free GSK-3β" for inhibition

GSK-3β and its role in insulin signaling

One of the primary functions of GSK-3β is to phosphorylate and inactivate the enzyme glycogen synthase (GS) (Parker et al., 1983; Roach, 1990, Zhang et al., 1993). GSK-3β is constitutively active in cells and insulin mediated regulation/inhibiton of GSK-3β leads to the activation of glycogen synthase. In the presence of insulin, sequential activation of Insulin Receptor Substrate (IRS-1), PI3-kinase and Akt/PKB takes place, which eventually causes inactivation of GSK-3β (Sutherland et al., 1993; Cross et al., 1994, 1995; Stambolic and Woodgett, 1994). Akt/PKB mediates the GSK-3β inactivation by phosphorylating the N-terminal Ser-9 residue (Sutherland et al., 1993; Cross et al., 1994, 1995; Stambolic and Woodgett, 1994). Glycogen synthase and IRS-1 are important constituents of the insulin signaling pathway and both are putative substrates of GSK-3β enzyme (Rylatt et al., 1980; Parker et al., 1983; Liberman and Eldar-Finkelman, 2005). Like glycogen synthase; IRS-1 is phosphorylated and inactivated by GSK-3β thus imparing the insulin signaling pathway (Eldar-Finkelman and Krebs, 1997; Liberman and Eldar-Finkelman, 2005). GSK-3β plays a critical role at multiple steps in the insulin signaling pathway. Various studies indicate that GSK-3β acts as a negative modulator of insulin signaling and plays an important role in maintaining glycogen synthase in a repressed state. Hence deregulation or hyper activation of GSK-3β has been implicated in the development of diabetes mellitus type 2 (formerly known as non insulin dependent diabetes mellitus – NIDDM) (Henriksen and Dokken, 2006).

GSK-3β and regulation of Hedgehog pathway

Hedgehog (Hh) signaling is involved in important cellular functions like cell division and cell survival and its deregulation is linked to the development of different disorders (reviewed in Murone et al., 1999; Briscoe and Pascal, 2013). Hedgehog signaling is a complex pathway and GSK-3β plays an important role in it along with other kinases (Jia et al., 2002; Jiang et al., 1995;

Price and Kalderon, 2002, Lum et al., 2003). The downstream effector of the Hh signaling pathway in drosophila is called as Cubitus interruptus (Ci) and it is responsible for the regulation of target genes in response to Hh stimulation. (Alexandre et al., 1996; Von Ohlen et al., 1997; Methot and Basler, 2001). Ci appears in two forms called as Ci_{155} and Ci_{75} (a truncated form of Ci_{155}) and in relation to the regulation of Hh target genes - Ci_{155} is the active form and Ci_{75} is the repressor form (Aza-Blanc et al., 1997). GSK-3β is involved in the conversion (truncation) of Ci_{155} into its repressor form along with other kinases. In the absence of Hh stimulation or Hh ligands, Protein Kinase A (PKA) phosphorylates Ci thus priming it for further phosphorylation by GSK-3β and caesin Kinase 1δ (CK1δ) followed by the partial proteasomal degradation to yield Ci_{75} (Jia et al., 2002; Jiang et al., 1995; Price and Kalderon, 2002). In vertebrates the final downstream effector of the Hh pathway is called as Gli and recently it was shown that PKA is involved in the multi site phosphorylation of Gli (Niewiadomski et al., 2014). In the presence of Hh ligands the conversion of Ci into its repressor form i.e. Ci_{75} is inhibited (Methot and Basler, 1999). It can be summarized that Hh stimulation down regulates the activity of GSK-3β.

GSK-3β and diseases

Alzheimer's disease

Since GSK-3β plays a critical role in the regulation and stability of various important proteins, deregulation of GSK-3β has been associated with many diseases. There is enough evidence to directly link GSK-3β with the neuropathological mechanisms associated with Alzheimer's disease (AD). GSK-3β induces phosphorylation of microtubule associated tau protein, which resembles the Alzhemer's disease-like induced tau-phosphorylation. (Hanger et al., 1992; Yang et al., 1993; Woods et al., 2001). Increased levels of GSK-3β have been found in AD compared to normal human brains and immunohistochemical studies have detected GSK-3β

in the neurofibrillar tangles of the AD brain tissue (Yamaguchi et al., 1996; Imahori and Uchida, 1997; Pei et al., 1997, 1999). These studies indicate that GSK-3β has a role to play in the etiology of Alzheimer's disease.

Insulin resistance and diabetes mellitus type 2

Role of GSK-3β in the development of insulin resistance and diabetes mellitus type 2 has been established in various studies (Henriksen and Dokken, 2006). Elevated GSK-3β has been implicated in the development of insulin resistance in skeletal muscle cells. Increased levels of GSK-3β is found in the tissues of insulin resistant obese rodent models like high fat fed mice (Eldar-Finkelman et al., 1999), obese zucker rats (Dokken et al., 2005). Enhanced GSK-3β is also found in type 2 diabetic humans (Nikoulina et al., 2000). At the molecular level it has been observed that GSK-3β can hamper insulin signaling by interfering with the important components of the insulin signaling pathway. GSK-3β can phosphorylate Ser-332 of IRS-1*in vitro* which could hinder its interaction or communication with insulin receptor (Liberman and Eldar-Finkelman, 2005; Aguirre et al., 2002). The disruption of interactions between insulin receptor and IRS-1 could have a detrimental effect on insulin signaling pathway and protect GSK-3β from the inhibitory action of insulin. GSK-3β phosphorylates glycogen synthase enzyme (GS) on multiple serine residues and leads to its inactivation (Parker et al., 1983; Roach, 1990, Zhang et al., 1993). Inactive glycogen synthase leads to decrease in glycogenesis (glycogen synthesis), which in turn would lead to an increase in the blood glucose levels (Cross et al., 1997; Summers et al., 1999). These observations demonstrate that deregulation of GSK-3β could play an important role in development of type 2 diabetes mellitus.

Cancer

GSK-3β is an important constituent of the β-catenin destruction complex along with APC (Adenomatous Polyposis Coli), axin, casein kinase 1 (CK1), protein phosphatase 2A (PP2A) and

E3-ubiquitin ligase β-TrCP (Zeng et al., 1997; Behrens et al., 1998; Hart et al., 1998, Ikeda et al., 1998;Itoh et al., 1998; Salic et al., 2000; Kikuchi, 1999). The GSK-3β mediated phosphorylation of β-catenin marks it for ubiquitin-mediated proteosomal degradation (Pefifer et al., 1994; Yost et al., 1996; Rubinfeld et al., 1996; Hart et al., 1996; Behrens et al., 1998; Oxford et al., 1997; Farr et al., 2000). GSK-3β and β-catenin are important members of the Wnt signaling pathway. Wnt activation results in the inhibition of GSK-3β (Bilic et al., 2007; MacDonald et al., 2009; Metcalfe and Bienz, 2011) leading to stabilization of β-catenin (Cook et al., 1996; Huber et al., 1996; Willert et al., 1999). The β-catenin translocates into the nucleus where it interacts with TCF/LEF proteins to form a complex; this complex binds to DNA and regulates the transcription of TCF/LEF target genes (Huber et al., 1996). Abnormal or constitutive repression of GSK-3β activity could lead to accumulation of β-catenin in the nucleus. Several studies have directly linked the de-regulation of β-catenin with development of various cancers (Smalley and Dale, 1999; Peifer and Polakis, 2000; Thakur and Mishra, 2013).

Regulation of GSK-3β

GSK-3β controls diverse cellular processes like gene expression, apoptosis, cell viability and development. Hence GSK-3β is regulated by complex regulatory mechanisms in order to avoid any unwanted consequences on the normal cellular functions. It has been observed that GSK-3β is regulated by multiple regulatory mechanisms.

Inhibitory Ser-9 phosphorylation of GSK-3β

The activity of GSK-3β is down regulated by an increase in the phosphorylation of its Ser-9 residue on the N-terminal region (reviewed in Plyte et al., 1992). Some GSK-3β inhibitors induce an increase in the Ser-9 phosphorylation of GSK-3β thereby inhibiting it (Frame and Cohen, 2001; Grimes and Jope, 2001; Doble and Woodgett, 2003). Phosphorylated Ser-9 residue on the

N-terminal tail of GSK-3β acts as a pseudo substrate thus blocking the access of the substrates to GSK-3β's catalytic site (Frame et al., 2001; Dajani et al., 2001). There are many different kinases that can phosphorylate GSK-3β at Ser-9 position depending on specific stimuli. Some of the kinases known to induce an increase in Ser-9 phosphorylation of GSK-3β are p70 S6 kinase (Armstrong et al.,2001; Krause et al., 2002; Terruzzi et al., 2002), p90Rsk (also called as MAPKAP kinase-1) (Brady et al., 1998; Saito et al., 1994), Akt (also called protein kinase B) (Sutherland et al., 1993; Shaw, et al., 1997; Cross et al., 1994, 1995; Stambolic and Woodgett, 1994), certain isoforms of protein kinase C (Ballou et al., 2001; Fang et al., 2002), and cyclic AMP-dependent protein kinase (protein kinase A) (Fang et al., 2000; Li et al., 2000; Tanji et al., 2002).

Insulin is a well characterized GSK-3β's indirect inhibitor and in response to insulin an increase in the inhibitory phosphorylation of the Ser-9 residue on the N-terminal region of GSK-3β is induced (Sutherland et al., 1993; Cross et al., 1995). The upstream kinases of the insulin signaling pathway play an important role in the insulin induced Ser-9 phosphorylation of GSK-3β (Sutherland et al., 1993; Welsh and Proud, 1993; Cross et al. 1995). Akt or protein kinase B is an important component of the insulin signaling pathway and it has been observed that in response to insulin, activation of Akt/PKB takes place. Activated Akt modulates an increase in the Ser-9 phosphorylation of GSK-3β (Sutherland et al., 1993; Shaw, et al., 1997; Cross et al., 1994, 1995; Stambolic and Woodgett, 1994). The negative regulation of GSK-3β activity via the Ser-9 phosphorylation is a crucial step in the receptor-coupled signaling processes. Much effort has been directed to identify the specific kinases used by different receptor coupled signaling systems. Different kinases activated in response to different stimuli leads to the inhibitory Ser-9 phosphorylation of GSK-3β. However the Ser-9 phosphorylation of GSK-3β is reversible and its dephosphorylation is mediated by protein phosphatase 1 (PP1) (Zhang et al., 2003).

Regulation of GSK-3β activity by protein complex formation

Apart from the inhibitory Ser-9 phosphorylation, GSK-3β is regulated by the formation of a protein complex mediated by GSK-3β binding proteins. Regulation of GSK-3β by protein complex formation is a complicated mechanism which involves proteins like GSK-3β binding protein (GBP) (Yost et al., 1998; Farr et al., 200), Axin (Ikeda et al., 1998; Yamamoto et al., 1999) and the axin related protein named as Axil (Yamamoto et al., 1998) or Conductin (Behrens et al., 1998). Presently FRAT1 and FRAT2 are included in the GBP family and regulate GSK-3β by forming a protein complex with it (Jonkers et al., 1997; Li et al., 1999; Thomas et al., 1999; Yost et al., 1998). An interesting fact about binding of GBP to GSK-3β is that it doesn't inhibit the catalytic activity of GSK-3β towards all its substrates (Thomas et al., 1999). According to Thomas et al binding of FRAT1 to GSK-3β inhibits its activity towards β-catenin but not glycogen synthase. The binding of GBP family proteins to GSK-3β produces a probable localized inhibition of GSK-3β enzyme, partially limiting its phosphorylating activity towards certain substrates.

As explained GSK-3β forms the β-catenin destruction complex along with axin, APC (Adenomatous Polyposis Coli), casein kinase 1 (CK1), protein phosphatase 2A (PP2A) and E3-ubiquitin ligase β-TrCP (Hart et al., 1996; Behrens et al., 1998; Oxford et al., 1997). β-catenin is an important substrate of GSK-3β and the GSK-3β mediated phosphorylation of β-catenin marks it for ubiquitin-mediated proteosomal degradation (Behrens et al. 1998; Oxford et al. 1997). Axin and APC both are substrates of GSK-3β and phosphorylation of axin and play an important role in the assembly of the β-catenin destruction complex (Rubenfeld et al., 1996; Ikeda et al., 1998). The GSK-3β of the β-catenin destruction complex induces an increase in the rate of β-catenin phosphorylation thus marking it for proteasomal degradation (Rubinfeld et al., 1996; Hedgepeth et al., 1999; Farr et al., 2000). The relationship between GSK-3β and axin is mutualistic. Binding of axin enhances the activity of GSK-3β similarly the GSK-3β induced phosphorylation of axin

stabilizes it from degradation (Yamamoto et al., 1999). There is evidence to support that GBP and axin may not bind to GSK-3β simultaneously but compete with one another for protein complex formation (Farr et al., 2000). One such regulatory mechanism is elicited by Disheveled (Dsh) proteins. Dsh is a negative regulator of GSK-3β, which can bind both to FRAT1 and axin. Dsh facilitates the binding of FRAT1 to GSK-3β and simultaneously enhances the disassociation of axin from GSK-3β (Li et al., 1999; Krylova et al., 2000).

The differential regulation of GSK-3β by protein complex formation indicates that there are two populations of cellular GSK-3β, the "free GSK-3β" and the axin "bound GSK-3β", which is part of the β-catenin destruction complex (**refer schematic diagram 2 on page 14**). There is evidence to suggest that the free and bound GSK-3β could be participating in independent and different signaling pathways (Ding et al., 2000) (discussed in more detail in chapter 5).

Competitive inhibition

GSK-3β is a Mg^{2+} and ATP dependent enzyme. Any interference with the binding of Mg^{2+} ions and ATP to the GSK-3β enzyme will restrict its kinase activity. Some of the GSK-3β inhibitors act either by competing with Mg^{2+} or ATP or both thus inhibiting the enzyme. (explained in Table 3)

GSK-3β inhibitors

The most desired characteristics in an inhibitor are its specificity and potency towards the target proteins. There are various classes of GSK-3β inhibitors and Table 3 provides a comprehensive list of different GSK-3β inhibitors and their specificity towards other kinases. The simplest among the various classes of GSK-3β inhibitors are the metal cation group.

Lithium – an efficient GSK-3β inhibitor

Lithium ($Li^{+)}$ is a monovalent metal cation that acts as an efficient inhibitor of GSK-3β. Li^{+} is the most well characterized metal cation inhibitor of GSK-3β and is commonly used in the form of LiCl salt (Klein and Melton, 1996, Stambolic et al., 1996). Li^{+} inhibits the activity of GSK-3β both directly and indirectly (Jope, 2003). GSK-3β is a Mg^{2+} dependent enzyme and Li^{+} inhibits GSK-3β by directly competing with Mg^{2+} ions for the magnesium binding sites (Ryves and Harwood, 2001; Ryves et al., 2002). Like insulin, Li^{+} too leads to an increase in the inhibitory Ser-9 phosphorylation in a wide variety of cells thereby inhibiting GSK-3β enzyme indirectly (Zhang et al., 2003). According to *Zhang et al* Li^{+} induces an increase in the Ser-9 phosphorylation of GSK-3β by perturbing the GSK-3β-TIMAP-PP1 feed back loop. The Li^{+} induced inhibition of protein phosphatase 1 (PP1) leads to a build up of pSer-9-GSK-3β thus accounting partly for the regulation of GSK-3β.

From the inhibitory Ser-9 phosphorylation perspective, Li^{+} mimics insulin and has a positive effect on glycogen metabolism (Cheng et al., 1983; Bosch et al., 1986; Woo et al., 2000). In addition Li^{+} also mimics Wnt ligands and activates the Wnt signaling pathway, which in turn leads to nuclear localization of β-catenin (Stambolic et al., 1996; Hedgepeth et al., 1997). Li^{+} is a reasonably selective GSK-3β inhibitor but its inhibitory concentration range is in millimolar (Ki = 2 mM) (Klein and Melton, 1996). Beryllium (Be^{2+}) another metal cation inhibitor of GSK-3β is 1000 times more potent compared to lithium (Mudireddy et al., 2014). But not much is known about the role of Be^{2+} as a GSK-3β inhibitor.

Table 3a. List of GSK-3β inhibitors

(Adapted from - Cell and Molecular Life Sciences, volume - 64, 2007, 1930-1944, Glycogen synthase kinase 3: a key regulator of cellular fate, Forde J.A, Dale T,Table.1 GSK-3 inhibitors; with kind permission from Springer Science and Business Media")

(Adapted with permission from Journal of Cell Science, Doble BW, Woodgett JR. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* **116**: 1175-1186.)

Inhibitor	Specific example	Inhibition potency	Mode of action	Specificity	Reference
Lithium	N/A	Ki = 2 mM	Competes with Mg^{2+}, Mimics Wnt signaling	Also inhibits IMPase, Fructose 1,6 biphosphate, IPPase, BPNT, Phosphoglucomutase (PGM)	Ryves and Harwood, 2001; Klien and Melton, 1996; York et al., 2001; Gurvich and Klein, 2002; Ray et al., 1978
Bivalent Zinc	N/A	IC_{50} = 15 µM	Undetermined; doesn't compete for substrate	N/A	Ilouz et al., 2002
Beryllium	N/A	IC_{50} = 6 µM	Competes with Mg^{2+} and ATP	Also inhibits cdc 2	Ryves et al., 2002
Anilino maleimides	SB216763 and SB415286	IC_{50} = 10 – 30 nM	ATP competitor	Doesn't inhibit a range of other kinases	Couglan et al., 2000; Smith et al., 2001
Bisindole malemides	Ro 31-8220, GF 109203x	IC_{50} = 5 – 170 nM	ATP competitor	Also inhibits PKC	Hers et al., 1999

Table 3b. List of GSK-3β inhibitors

Inhibitor	Specific example	Inhibition potency	Mode of action	Specificity	References
Bisindole malemides	Ro 31-8220, GF 109203x	IC_{50} = 5 – 170 nM	ATP competitor	Also inhibits PKC	Hers et al., 1999
Aldisine alkaloids	hymenialdisine	IC_{50} = 5 – 10 nM	ATP competitor	Also inhibits MEKs, CK1 and CDKs	Meijer et al., 2000; Tasdemir et al., 2002
Aloisines	aloisine A	IC_{50} = 0.4 – 85 µM	ATP competitive	Also inhibits CDK1/cyclin B and CDK5	Mettey et al., 2003
Indirubins	6-bromoindirubin-3'-oxime aka **BIO**	IC_{50} = 5 – 50 nM	ATP competitor	Also inhibits CDKs	Meijer et al., 2003; Leclerc et al., 2001
Paullones	Alsterpaullone	IC_{50} = 4 – 80 nM	ATP competitor	Also inhibits CDKs and mMDH	Knockaert et al., 2002; Leost et al., 2002
Pseudo substrate peptide	N/A	Ki = 0.7 mM	Substrate competitor	Specific	Dajani et al., 2001

GSK-3β: A therapeutic target

In recent times GSK-3β has emerged as an important therapeutic target because of its role in the etiological development of different abnormalities and diseases. It is important to identify potent inhibitors of GSK-3β so as to characterize and understand the impact of GSK-3β deregulation. GSK-3β inhibitors could have therapeutic potential in the pathophysiological process involving hyper active GSK-3β. For example in patients suffering from diabetes milletus type 2 and neurological disorders; inhibitors of GSK-3β could serve the purpose of a potential therapeutic agent. It has also been observed that inhibition of GSK-3β leads to an increase in cellular senescence, apoptosis and sensitization of tumor cells to ionizing radiation (Thotala et al., 2008) and chemotherapeutic agents (Miyashita et al., 2009).

It has been reported that the levels and activity of GSK-3β are elevated in diabetic and obese mice (Elder-Finkelman et al., 1999). There is a great pharmaceutical interest in identifying compounds which can mimic insulin and repress GSK-3β activity especially in patients suffering from type 2 diabetes. GlaxoSmith Kline developed GSK-3β inhibitors belonging to the malemide group and these compounds facilitated the activation of glycogen synthase in liver cells (Coghlan et al., 2000).

Li^+, another GSK-3β inhibitor, is already in use as a mood stabilizer. Li^+ treatment leads to slight stimulation of the glucose uptake as well as an increase in the translocation of glucose transporter GLUT4 to the plasma membrane in 3T3-L1 and rat adipocytes (Chen et al., 1998; Orena et al., 2000; Summers et al., 1999), indicating the potential therapeutic role the GSK-3β inhibitors could play.

The use of GSK-3β inhibitor for therapeutic applications sounds exciting and promising but it also raises serious concerns about the unwanted outcomes, which could result from the inhibition of GSK-3β. One of the primary concerns is the specificity of GSK-3β inhibitors, majority of the

GSK-3β inhibitors seems to be regulating other related kinases as well (explained in Table 3). A more serious problem that could arise due to inhibition of GSK-3β is the stabilization and nuclear localization of β-catenin. The abnormal accumulation of β-catenin due to different reasons is associated with the development of various cancers especially colorectal cancers (Thakur and Mishra, 2013). It has been reported that GSK-3β inhibitors developed by GlaxoSmith Kline leads to activation of glycogen synthase but also causes dramatic increase in the levels of β-catenin (Cross et al., 2001). Use of the GlaxoSmith Kline GSK-3β inhibitors also stimulates transcription of genes regulated by β-catenin (Coghlan et al., 2000). However whether prolonged use of GSK-3β inhibitors stimulates the formation of tumor or enhances tumorigenesis in animal models is still under investigation. There is always a possibility that prolonged use of GSK-3β inhibitors could be potentially oncogenic.

Taking into consideration the adverse effects elicited due to GSK-3β-inhibiton induced nuclear localization of β-catenin, a pathway specific GSK-3β inhibitor is desired. A good GSK-3β inhibitor, which can be used for therapeutic purpose would be the one that can inhibit the non-axin or free GSK-3β. The selective inhibition of non-axin GSK-3β should not have any effect on the canonical Wnt/ β-catenin pathway since it is the axin-bound GSK-3β, which is involved in the regulation of β-catenin.

Beryllium: An unsung anti-hero

Beryllium in the form of $BeSO_4$ salt acts as a potent GSK-3β inhibitor and is less toxic to some cells at physiologically effective concentrations, compared to LiCl (Mudireddy et al., 2014). Apart from the fact that Be^{2+} could be competing for both Mg^{2+} and ATP binding sites on GSK-3β, not much is known about the mechanism by which Be^{2+} inhibits the activity of GSK-3β (Ryves et al., 2002).

Beryllium (Be) is an alkaline earth metal belonging to the group IIA elements. The atomic number of beryllium is 4 and it occurs in two oxidation states, Be^0 and Be^{2+}. Beryllium has many industrial applications, since it is lighter than aluminium yet stronger than steel. Beryllium was discovered by Nicholas-Louis Vauquelin in the year 1797. The French chemist AntoineAlexandre-Brutus Bussy and German chemist Friedrich Wohler isolated beryllium independently in the year 1828 (de Laeter et al., 2003). Out of the nine known radioisotopes of beryllium, beryllium-7 (Be-7) and beryllium-10 (Be-10) are stable with half lives of 53.29 days and $1.51x10^6$ years respectively (Hammond, 2000).

The cytostatic effects of beryllium were first documented in 1949, using an animal limb regeneration model (Thornton, 1949; Chevremont and Firket, 1951). Apart from the study published by Ryves *et al*, no work has been published to examine beryllium's role as GSK-3β inhibitor until it was demonstrated by our lab that beryllium is a 1000 times more potent GSK-3β inhibitor (Mudireddy et al., 2014). Traditionally the aim of the research work related to beryllium has been to analyze/establish its cytotoxic effects. Beryllium has been classified as a potent carcinogen in humans and in rats. Various studies in rats have shown that beryllium when inhaled caused a high incidence of lung tumors (Haley et al., 1990; Nikula et al., 1997; Finch et al., 1998).

The route of beryllium administration plays a crucial role in inducing its toxic effects.

Acute oral toxicity

Oral toxicity of beryllium was tested in rats, where in the LD_{50} was found to be >2000 mg kg^1 body weight (Strupp, 2011). The very high dosage of beryllium to induce its lethal effects when administered orally suggests that ingestion of soluble form of beryllium is not very harmful.

Inhalation toxicity

The available literature suggests that delayed toxic effects after acute inhalation of beryllium metal has been reported in rats (Haley et al., 1990; Nikula et al., 1997; Finch et al., 1998). It has been observed that inhalation of high doses of soluble form of beryllium is known to cause an acute beryllium disease, which is an obstructive inflammatory lung disease (Eisenbud, 1955; Cummings et al., 2009).

Dermal sensitization

Studies have shown that beryllium did not lead to any dermal sensitization reaction in patients who had a history of beryllium sensitization in beryllium plants (Curtis, 1951).

Cytotoxicity and carcinogenicity

In our lab it was observed that beryllium induces cytotoxicity only when used at high concentrations (high micromolar range). Different cell lines are fairly resistant to the low beryllium dosage (10 – 100 µM). Majority of the research work to establish the role of beryllium as a carcinogen has been carried out in rats. A robust carcinogenic response is observed in rats after inhalation of beryllium metal at high concentrations. Experimental attempts to reproduce the same results in mice and guinea pigs showed no carcinogenic response (Schepers, 1961). It has been reported that the potential of beryllium induced lung cancer increases only when the patients are exposed to high concentrations (Hollins et al., 2009). In an independent study it was observed that exposure of BALB/c-3T3 cells to high concentrations of $BeSO_4$ (100 – 200 µg/ml or 0.5 – 1 mM) caused an increase in the transformation efficiency (Keshava et al., 2001). From the epidemiological studies it can be speculated that beryllium is hazardous when inhaled at high dosage. The studies indicating the carcinogenic role of beryllium has been mostly centered on the use of beryllium metal. It was observed that beryllium when inhaled in the form of beryllium metal or beryllium oxide caused lung cancer in rodents (Gordon and Bowser, 2003). However

there is no evidence to suggest that beryllium in form of salt could be a potentially strong carcinogen.

The probable carcinogenic role of particulate beryllium cannot undermine the potential of beryllium salt as a potent GSK-3β inhibitor. In our study we have used beryllium in the form of $BeSO_4.4H_2O$ and investigated the intracellular effects of beryllium treatment at molecular level in different cell lines. The focus of this study was to investigate beryllium as a biologically relevant GSK-3β inhibitor. We also tried to understand the inhibitory mechanisms that play an important role in the beryllium induced inhibition of GSK-3β.

CHAPTER 3

Dose Response Curve – survey of different cell lines to analyze their sensitivity towards $BeSO_4$

Introduction

The aim of this study is to analyze and document the cytostatic/cytotoxic effects of Be^{2+} on different mammalian cell lines. Cell lines selected for this study were cultured in media supplemented with Be^{2+} in the form of $BeSO_4.4H_2O$. It is imperative to have prior knowledge about the behavior of different cell lines when cultured in the presence of beryllium because cell signaling studies could be highly cell type specific. Having a good idea about the sensitivity of different cell line towards $BeSO_4$ will help us in selecting the correct cell line. Some cell lines could be highly sensitive or highly resistant to $BeSO_4$ making them unsuitable for the present study. It was observed that beryllium caused an increase in the cell division time or doubling time of HFL-1 cells (human lung fibroblasts) (Absher et al., 1983). Treatment with beryllium seems to be inducing diametrically opposite effects on cells. Beryllium is known to promote carcinogenesis (Wagoner at al., 1980) implying that it is capable of inducing unregulated cell proliferation. It has also been demonstrated that beryllium induces cytostatic effects in the form of cellular senescence indicating that beryllium can effectively regulate cellular growth (Coates et al., 2007; Gorjala and Gary, 2010).

In order to analyze the sensitivity of different cell lines towards $BeSO_4$ a "dose response curve" was obtained. Different mammalian cell lines are cultured in the presence of beryllium salt over a period of nine days. The cell numbers are monitored on day3, 6 or 9. The effect of beryllium on the survival of mammalian cells can be quantified form the cell counting experiment

(DRC curves). This survey includes the dose response curve data of 8 different mammalian cell lines. This survey will provide an insight into the senstivity of mammalian cells towards $BeSO_4$.

Materials and methods

Cell lines

Cell lines B35, C6, Caki-1, IMR 32, NIH/3T3, MCF-7, T47D were purchased from ATCC (Manassas, VA). The SF539 cell line was purchased from National Cancer Institute.

Cell culture media

RPMI 1640 HEPES (cat#23400-021, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic-mycotic was used for culturing B 35, C6, Caki-1, IMR 32 and SF539 cell lines. Only for NIH/3T3 cells, RPMI 1640 was supplemented with 10% bovine growth serum (BGS) instead of FBS. For MCF-7 and T47D cell line the RPMI 1640 was supplemeted with growth factors – 344.3 pM insulin and 1 nM β-estradiol.

Dose response curve - cell counting

Cells were cultured in 60 mm CELLSTAR cell culture dishes (cat#664 160) at 37˚C using a 5% CO_2 incubator. Cells were dosed with culture media supplemented with appropriate concentrations of $BeSO_4$ and the dosing day was counted as Day 0. On Day 3 the cells were collected by trypsinization using 0.5 ml trypsin (0.05% Trypsin-EDTA). The cells were collected by adding 2.5 ml of RPMI (3.5 ml RPMI + 0.5 ml trypsin = total 3 ml cell suspension). Cells were counted using a cell counter .0.5 ml of the total cell suspension from day 3 was added to a new 60 mm culture dish. The cells were allowed to grow till day 6 and were trypsinized again, counted in a manner similar to day 3. 0.5 ml of cells from the total 3 ml cell suspension from day

6 was added to a new 60 mm dish. The process was repeated again on Day 9 with the exception that the cells were discarded instead of re seeding.

Note: Cells have to be seeded in the range of 1.5–2.0 x 10^6 cells/100 mm dish to obtain sub confluency (50-60%) after approximately 12 hr incubation

Data analysis

The day 3, day 6 and day 9 cell counts were fitted onto a plot using non-linear regression. The effect of $BeSO_4$ on the different cell lines was represented as % of control (untreated cells) on the y-axis and the log values of $BeSO_4$ concentration on the x-axis. GraphPad Prism v6.0c (Mac OS) was used for curve fitting and calculating IC_{50} values.

Results

Beryllium in the form of $BeSO_4.4H_2O$ was used at concentrations ranging from 0, 0.3, 1, 3, 5, 10, 30, 100, 300, 500, 1000 or 3000 µM. The IC_{50} values were calculated using the day 6 data. The day 3 and day 9 DRC data was not used for calculating the IC_{50} values because the beryllium resistant cell lines may not show optimum response by day 3. The 9 day $BeSO_4$ treatment could be extremely stringent on the $BeSO_4$ sensitive cell lines hence the day 6 data was used to calculate the IC_{50} values.The results in Fig. 1a, Fig. 1b and Fig. 1c demonstrates that all cell lines do not respond uniformly to $BeSO_4$. Some of the cell lines are sensitive to beryllium while others are resistant. The results have been summarized in table 4.

A

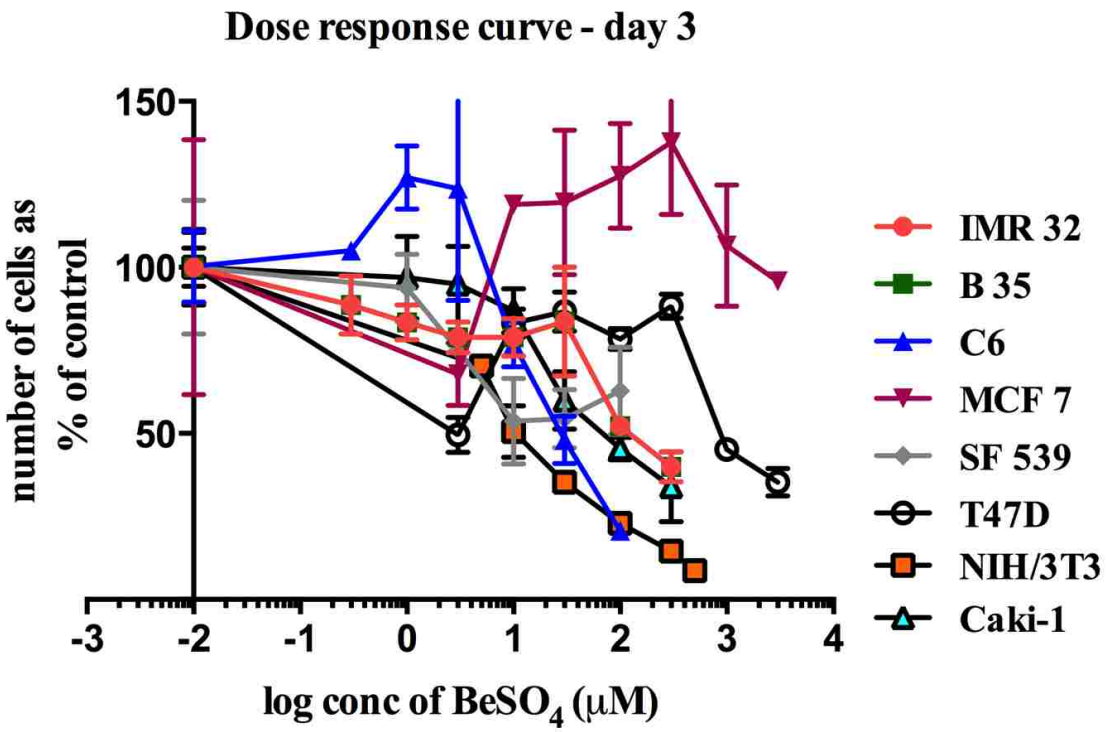


Fig. 1a Day 3 dose response curve. Each curve represents the response of individual cell lines to concentrations of $BeSO_4$ ranging from 0 – 3000 μM on day 3. The day 3 data was not used for calculating the IC_{50} values because the cyto toxic effect of $BeSO_4$ may not manifest correctly at a short exposure time i.e. day 3, especially for $BeSO_4$ resistant cells lines.

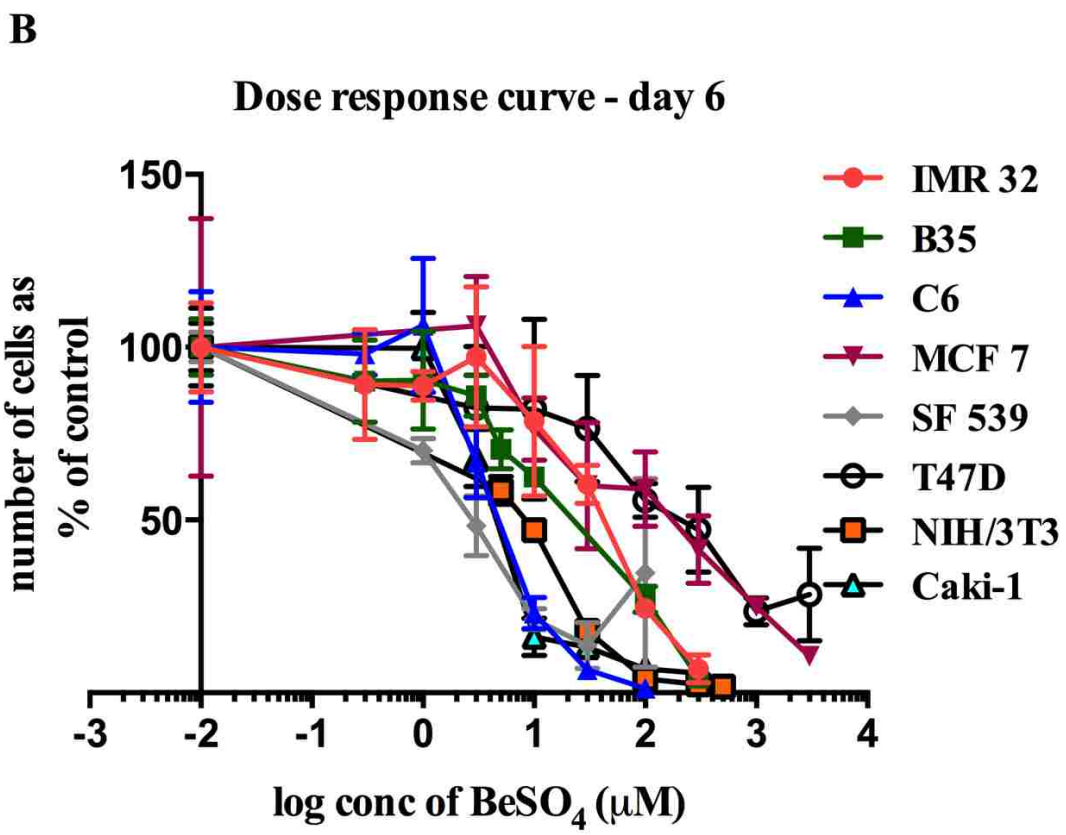


Fig. 1b Day 6 dose response curve. Each curve represents the response of individual cell lines to concentrations of $BeSO_4$ ranging from 0 – 3000 μM on day 6. The day 6 data was used to calculate the IC_{50} values. Day 6 data was selected because by day 6 the cells are not under exposed nor over exposed to the inhibitory cytotoxic/cytostatic effects of $BeSO_4$.

C

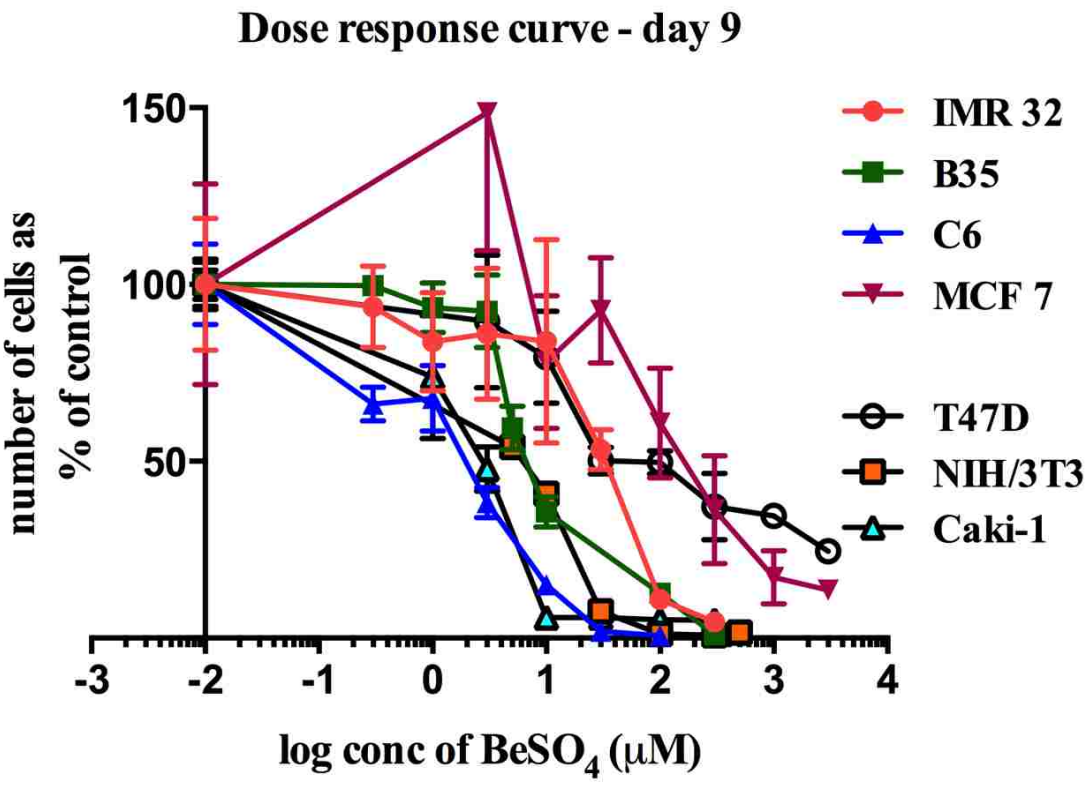


Fig. 1c Day 9 dose response curve. Each curve represents the response individual cell lines to concentrations of $BeSO_4$ ranging from 0 – 3000 μM on day 9. The possibility of cells being over exposed to $BeSO_4$ might interfere with the correct IC_{50} calculation. The day 9 data was not used to calculate the IC_{50} values.

Table 4. IC_{50} values of $BeSO_4$ for different cell lines

	Cell Line	IC_{50} value (µM $BeSO_4$)
1	B 35	34.50
2	C6	5.27
3	Caki-1	4.93
4	IMR 32	37.94
5	MCF-7	136.1
6	NIH/3T3	7.59
7	T47D	190.9
8	SF 539	2.9

Discussion

The DRC analysis clearly shows that not all cell lines respond uniformly to $BeSO_4$. In this study the T47D cell line was found to be the most resistant to $BeSO_4$ followed by MCF-7, IMR 32 and B35. SF539 was found to be the most sensitive towards $BeSO_4$ followed by C6, Caki-1 and NIH/3T3. In order to investigate the intra cellular effects of beryllium on mammalian cells it is essential to choose a cell line that is not too resistant to the beryllium salt. If a cell line is too resistant to beryllium treatment then it could be possible that the particular cell type may not be a good system to study the beryllium induced intra cellular effects. If a cell line is too sensitive to beryllium salt treatment then it would be difficult to recover sufficient number of viable cells after Be^{2+} treatment for further processing (for example – generating western samples). Hence cell lines that can fairly tolerate beryllium treatment are usually selected for further studies.

We have included cell lines of different lineages in our survey Table 4 & 5. It is observed that cell lines of mammary lineage (MCF-7&T47D) seems to be resistant to higher concentration of beryllium salt with an IC_{50} value range of approximately 150 µM (Table 4). In an independent study it was observed that RKO cells (human colon carcinoma) were found to be resistant to higher concentrations of beryllium with an IC_{50} value of 440 µM (Gorjala, 2012).

GSK-3β plays an important role in the regulation of microtubule associated neuronal protein tau. Tau protein is involved in several functions associated with neurons (Liu et al., 1999; Avila et al., 2004; Fuster-Matanzo et al., 2009) GSK-3β induces phosphorylation of tau protein that resembles the Alzhemer's disease-like induced tau-phosphorylation (Hanger et al., 1992; Yang et al., 1993; Woods et al., 2001). Tau protein seems to be playing an important role in cell lines that are of neuronal lineage. Hence we included neuronal cell lines like B35, C6, IMR-32 and SF-539 in our study for potential use in future work related to GSK-3β-tau protein. It was observed that the neuronal cell lines can tolerate optimum concentration of $BeSO_4$ (IC_{50} values 3 – 30 µM).

Independently it was also observed that additional neuronal cell lines A172 and U87MG (Gorjala, 2012) also showed a similar trend in their response to beryllium treatment as observed in our study.

It can be summarized that cell lines of mammary and colorectal lineage included in our study seems to be resistant to concentrations higher than 100 µM of $BeSO_4$. The lone cell line representing the human kidney cells were found to be sensitive to $BeSO_4$ (IC_{50} = 4.93 µM). However cell lines representing neuronal lineage seem to be respond to a range of different concentrations of $BeSO_4$ For example SF-539 was found to be very sensitive to Be^{2+} treatment (IC_{50} = 2.9 µM). However, most of the other neuronal cell lines like C6, A172 and U87MG were found to be responsive to optimum concentrations of $BeSO_4$ (approx 10 µM).

The rate of $BeSO_4$ uptake by these cell lines could be one of the possible reasons for their differential sensitivity towards $BeSO_4$. ICP MS analysis could provide an idea as to which cell lines fail to uptake $BeSO_4$ from the surrounding media.

Table 5 General information about cell lines used in this study (www.atcc.org)

S. No	Cell Line	ATCC info	Species	Origin, Type	TP53 status	Refrences
1	B35	CRL-2754	*Rattus norvegicus*	Neuronal, neuroblast	NA	NA
2	Caki-1	HTB-46	*Homo sapiens*	Kidney, clear cell carcinoma	Wild type	Jia et al., 1997.
3	C6	CCL-107	*Rattus norvegicus*	Neuronal, glioma	Wild type	Asai et al., 1994.
4	IMR-32	CCL-127	*Homo sapiens*	Neuronal, neuroblast	Wild type	NA
5	MCF-7	HTB-22	*Homo sapiens*	Breast, adenocarcinoma	Wild type	Wasielewski et al., 2006.
6	T-47D	HTB-133	*Homo sapiens*	Breast, Ductal carcinoma	Mutated	Nigro et al., 1989.
7	NIH/3T3	CRL-1658	*Mus musculus*	Embryo, fibroblast	NA	
8	SF539	NCI-60 cell line*	*Homo sapiens*	Neuronal, Glioma	Mutated	Forbes et al., 2010.
9	A172	CRL-1620	*Homo sapiens*	Neuronal, Glioma	Wild type	Mirzayans et al., 2005
10	U87MG	HTB-14	*Homo sapiens*	Neuronal, Glioma	Wild type	Van Meir et al., 1994.
11	HFL-1	CCL-153	*Homo sapiens*	Fetus lung, fibroblast	NA	NA

CHAPTER 4

Beryllium a potent GSK-3β inhibitor

Introduction

Beryllium (Be^{2+}) is a metal cation inhibitor of GSK-3β. The other metal ions which are included under this group are lithium (Li^{+}) and zinc (Zn^{2+}). Lithium is the most well characterized metal cation inhibitor of GSK-3β, which mimics both insulin (Cheng et al., 1983; Bosch et al., 1986; Woo et al., 2000) and Wnt ligands (Stambolic et al., 1996; Klein and Melton, 1996; Hedgepeth et al., 1997). The effects of lithium treatment on GSK-3β at molecular level and the underlying inhibitory mechanisms have been studied by different research groups. Some of the important points related to lithium's role as GSK-3β inhibitor can be summarized as follows:

- Li^{+} treatment leads to an increase in the inhibitory Ser-9 phosphorylation of GSK-3β (Zhang et al., 2003).
- Li^{+} mimics Wnt ligands and activates the Wnt signaling pathway (Stambolic et al., 1996; Hedgepeth et al., 1997).
- Ryves and Hartwood reported that Li^{+} competes with Mg^{2+} ions for the Mg^{2+}- binding sites of GSK-3β enzyme.
- Li^{+} mimics the GSK-3β-inhibitory action of both insulin and Wnt ligands (Cheng et al. 1983; Bosch et al. 1986; Woo et al. 2000; Stambolic et al., 1996)

Li^{+} is a well characterized GSK-3β inhibitor with a K_i value of 2 mM (Klein and Melton, 1996). Li^{+} is a fairly specific inhibitor of GSK-3β but it also inhibits other kinases (explained in Table. 3). Be^{2+} is a GSK-3β inhibitor eliciting its GSK-3β - inhibitory action at a much lower concentration compared to the Ki value of Li^{+} (Mudireddy et al., 2014). The IC_{50} value of Be^{2+} is 6

μM, which is much lower than the IC_{50} value of Li^+ required for GSK-3β inhibition (Ryves et al., 2002). This preliminary study is to investigate whether Be^{2+} is a better and potent GSK-3β inhibitor compared to the other metal ion Li^+.

There is a great deal of information explaining the role/mechanism of Li^+ as a GSK-3β inhibitor but not much is known about Be^{2+}. Except for the information that Be^{2+} competes for both Mg^{2+} and ATP binding sites on GSK-3β nothing much is known about its inhibitory effects on GSK-3β. There are many unanswered questions about beryllium's role as a potent GSK-3β inhibitor.

- Can beryllium inhibit the activity of GSK-3β?
- How potent/efficient is beryllium compared to lithium in terms of GSK-3β inhibition?
- Is beryllium a specific GSK-3β inhibitor?
- What could be the underlying inhibitory mechanism behind beryllium induced inhibition of GSK-3β?

In this study we investigated the effect of Be^{2+} on the kinase activity of pure recombinant GSK-3β enzyme *in vitro*. In this study we also analyzed the effect of Be^{2+} on treated cells and emphasis was laid to investigate the effect of Be^{2+} on the viability of cells compared to Li^+.

As explained in the Chapter 2, Ser-9 phosphorylation of GSK-3β plays an important role in its regulation. We investigated whether Be^{2+} can regulate the Ser-9 phosphorylation of GSK-3β.

Independently, the effect of Be^{2+} on endogenous GSK-3β was also analyzed as part of this research project (Mudireddy et al., 2014) but is not part of the thesis.

Materials and methods

Cell culture

HFL-1 (human lung fibroblast) and A172 (human glioblastoma) cells were obtained from ATCC (Manassas VA). Cells were grown in RPMI 1640 supplemented with 25 mM HEPES, 10% FBS, and 1x antibiotic-antimycotic (Invitrogen-Gibco) at 37˚C in 5% CO_2. HEPES was included as an auxiliary buffering agent.

Cell viability assay

ApoToxGlo Triplex assay (Promega-cat#G6321, lot# 32439) was used to assess the cellular protease activity in cultured cells. This assay simultaneously measures the activity of two cellular proteases generically called as the live cell protease and the dead cell protease. The live cell protease activity is present only in the intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC). The cell-permeant substrate enters the live intact cells where it is acted upon and cleaved by the live cell protease. The cleaved cell-permeant substrate generates a fluorescent signal proportional to the number of live cells. The other substrate in the assay is a fluorogenic cell-impermeant peptide substrate (bis-alanylalanyl-rhodamine 110; bis-AAF-R110). This cell-impermeant substrate is inaccessible to the live cell protease. The live cell protease becomes inactive upon loss of membrane integrity and leaks into the culture medium. The dead cell protease acts upon the second fluorogenic substrate and generates a fluorescent signal, which is different from the live cell signal. The live cell and dead cell proteases produce different fluorogenic products, AFC and R110 respectively. These products have different excitation and emission spectra allowing them to be detected simultaneously (Niles et al., 2007). A172 cells were cultured in the presence of different concentrations of beryllium and lithium for 24, 48 and 72 hr. The cells were initially cultured in 60 mm culture dishes and were trypsinized, counted and reseeded into 384 well plates

at 5000 cell/well. Proper care was taken to ensure that cells are under the influence of appropriate concentrations of the inhibitors for the above mentioned treatment periods. After the conclusion of the treatment time i.e at 24, 48 or 72 hr, the substrates from the ApoToxGlo Triplex (Promega) was added to the cells in the 384 well plates. The plate was then incubated for 30 min at 37˚C. The live cell (ex 400 nm, em 505 nm) and dead cell fluorescence (ex 485 nm, em 520 nm) was measured using a Tecan M1000 plate reader.

FRET based assay for analyzing in vitro kinase activity of enzymes

The z-Lyte is a FRET based biochemical assay that can be used to analyze the kinase activity of different enzymes. The assay kit provides customized substrate peptides that can be phoshorylated by the candidate enzyme whose kinase activity is to be tested. The z-lyte assay will be used to analyze the effect of $BeSO_4$ and LiCl on the activity of pure recombinant proteins - GSK-3β and PKA.

z-Lyte FRET assay principle:

The z-Lyte Ser/Thr peptide #9 (#PV3324) {sequence derived from glycogen synthase I (**PRPASVPP(pS)P(pS**) and the z-Lyte Ser/Thr peptide #1 (#PV3174) {sequence derived from porcine pyruvate liver kinase (**LRRASLG**)} were used as substrates for GSK-3β and PKA respectively. The peptide substrates are labeled with two fluorophores one at each end, which act as a FRET pair. In this case the peptide substrates are labeled with methyl coumarin (FRET donor) at one end and fluorescein (FRET acceptor) on the other end. The substrate peptides phosphorylated at the designated Ser residues will be immune to the peptide-cleavage activity of site-specific protease used in this assay. The substrate peptides phosphorylated at the designated Ser residues will not be cleaved and maintain the FRET pair thereby emitting a detectable fluorescence signal. Conversely the un-phosphorylated peptide substrate will be cleaved resulting in the loss of the FRET pair (loss of fluorescence signal as well)

Pure recombinant GSK-3β and PKA were used in the kinase assay at a final concentration of 1 ng/reaction in order to maintain the final or over all phosphorylation capacity of the enzyme at 50% rather than 100% (this was done avoid the saturation of enzyme activity). It is important to prepare the inhibitors in the kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% BRIJ-35, 100 µM ATP) only. The enzyme (1 ng/reaction) and the substrate peptides (2 µM – final concentration) were incubated for 60 min at 25℃ in a reaction volume of 20 µL. The kinase reaction was terminated by adding the development reagent A (site specific protease) and incubating the reaction mix for 60 min at 25℃, followed by the addition of the stop solution. The reaction mix is then transferred to a 96 well plate and the fluorescence is measured using Tecan M1000 platereader (coumarin fluorescence - ex 400 nm, em 445 nm) and fluorescein florescence - ex 400 nm, em 520 nm).

In the control samples (no inhibitors) the FRET pair will be intact because of the ability of the enzyme to phosphorylate the peptide substrates and shield them from the proteolytic activity of development solution A. The fluorescence values are expected to be higher for the control samples. If the inhibitors ($BeSO_4$ and LiCl) are successful in inhibiting the kinase activity of the candidate enzyme then the phosphorylation of the substrate peptide will be hampered. The unphosphorylated or under phosphorylated substrate peptides will be cleaved by the development reagent A. The cleavage of the substrate peptides results in the loss of the FRET pair that will eventually be translated in the form of low fluorescence signal values.

To validate the FRET-kinase assay, the effect of H-89 a PKA specific inhibitor was analyzed on purified recombinant GSK-3β and PKA enzymes.

Important precautions for z-Lyte assay:

- In order to maintain the phosphorylation levels at 50% the final enzyme concentration has to be approximately 1ng/reaction. (enzyme efficiency keeps decreasing with multiple thawing cycles).
- The $BeSO_4$ and LiCl stocks should always be prepared in the kinase buffer instead of any other solvent for best results.

Emission ratio = Coumarin Emission (445 nm)/Fluorescein Emission (520 nm)

The % of phosphorylation was calculated by using the formula provided in the assay kit protocol (Invitrogen #PV3324).

% Phosphorylation = 1- {(Emission Ratio * $F_{100\%}$) – $C_{100\%}$}/{($C_{0\%}$ - $C_{100\%}$) + [Emission Ratio *($F_{100\%}$ - $F_{0\%}$)]}

Emission Ratio = Coumarin/Fluorescein ratio of sample wells

$C_{100\%}$ = Average Coumarin emission signal of the 100% Phos. Control

$C_{0\%}$ = Average Coumarin emission signal of the 0% Phos. Control

$F_{100\%}$ = Average Fluorescein emission signal of the 100% Phos. Control

$F_{0\%}$ = Average Fluorescein emission signal of the 0% Phos. Control

Western blotting

Cells were cultured in 100 mm CELLSTAR cell culture dishes (cat#664 160). Total cell lysates were prepared from A172 and HFL-1 cells treated with $BeSO_4.4H_2O$ (lot&filling code: 413015/1 22001, Fluka) and LiCl (L-8895, Lot#22K0184, sigma) for 24 and 48 hr. MPER buffer (Prod#78501, thermo scientific) was used for protein extraction. The MPER buffer was supplemented with protease inhibitor (halt protease inhibitor cocktail kit, Pierce cat#78442) and phosphatase inhibitors (sodium fluoride - 20 mM, beta glycerol phosphate - 10 mM, sodium ortho

vanadate - 0.1 mM, paranitro phenyl phosphate - 20 mM and 1X EDTA). Protein concentration was measured by BCA assay (Thermo scientific, #23227) and the protein samples were normalized. Samples were run either on 10% SDS PAGE gel or 4-12% gradient SDS-PAGE gels (ref#WG1402BX10 from Novex-life technologies) and transferred onto a PVDF membrane 0.2 µm pore size (Millipore cat#IPFL20200; Bio-Rad. cat#162-0255). Post transfer the membranes were blocked in 10% milk or starting block TBS (Prod#37543, thermo scientific - when probing with phospho antibodies). The post transfer and blocked PVDF membranes were probed with the following primary antibodies separately: anti GSK-3α/β mouse monoclonal (cat#368662, clone 1H8, EMD Calbiochem), phospho-GSK-3β (Ser-9) affinity purified rabbit polyclonal antibody (cat#9336, Cell Signaling Technologies), phospho-GSK-3β (Ser-9) affinity purified mouse monoclonal antibody (cat#361527, clone 2D3, EMD-Calbiochem), p53 mouse monoclonal (cat#sc-126, clone DO1, Santa Cruz Biotechnology, actin goat polyclonal (cat#1615, Santa Cruz Biotechnology). Blots were incubated with the appropriate HRP conjugated secondary antibodies, developed with ECL-Plus (GE Healthcare Life Sciences), and imaged using a GE Typhoon 9410 Variable Mode Imager.

Antibody information

phospho GSK-3β (2D3) (Ser-9): Mouse monoclonal (#361527, EMD-Calbiochem) and goat anti-mouse IgG peroxidase (cat#A9917 Sigma Aldrich)

Total GSK-3α/β (1H8): Mouse monoclonal IgG_{2b} (cat#sc-368662, EMB – Calbiochem) and goat anti-mouse IgG peroxidase (cat#A9917, Sigma Aldrich)

p53 DO1: Mouse monoclonal IgG_{2b} (cat#sc-126 Santa Cruz Biotechnology, inc) and goat anti-mouse IgG peroxidase (cat#A9917, Sigma Aldrich)

Actin: Goat monoclonal IgG_{2b} (cat#sc-1615 Santa Cruz Biotechnology, inc) and bovine anti-goat IgG-HRP (cat#sc-2350, Santa Cruz Biotechnology, inc)

TR-FRET assay for quantifying the pSer-9 of GSK-3β

BacMam-enabled lantha screen cellular assay is an efficient tool to analyze the post translational modification of proteins. The LanthaScreen BacMam assay system (Invitrogen - life technologies) combines two powerful techniques to generate reliable fluorescence data with minimized background. It consists of different components - a modified baculo virus capable of infecting mammalian cells and a terbium (Tb) labeled antibody system. The modified bacculo virus acts a vector to accomplish the successful expression of a fusion protein (Green Fluorescent Protein + protein of interest) in the host cells. After the successful transduction of the host cells they are treated with the inhibitors or stimulators for a specific period of time. After the treatment cells are lysed and fresh cell lysates are probed with the Tb antibody specific for the post translational modification of the candidate protein (fusion protein).

In this study the LanthaScreen BacMam reagent (part#PM4355A0, lot#E0272-43882, Invitrogen - life technologies) was used to introduce a GFP-GSK-3β fusion protein (Green Fluorescent Protein–GSK-3β) into A172 cells. The fusion protein contains the GFP on the N-terminus, and the full length GSK-3β on the C-terminal side. The GFP and the GSK-3β are connected by a short linker. The successful transduction of the A172 cells with the BacMam virus can be confirmed by the expression of GFP. The transformed A172 cells numbering 40,000 each were then transferred to each well in a 384 well plate and cultured in the RPMI supplemented with $BeSO_4$ or LiCl for 24 hr. After the completion of the treatment time, cells were lysed using the LanthaScreen cellular assay lysis buffer (PM4355X, lot#MSN1143-086) supplemented with 3 nM of the Tb-conjugated anti-pSer9 GSK-3β antibody (PM4312AV, lot#MSN1023-183-3). Tecan M1000 plate reader was used to record the Tb fluorescence (ex 332 nm, em 485) and GFP fluorescence (ex 395 nm, em 515 nm). Phosphorylation was reported as ((515 nm/485 nm)/GFP Fluorescence)*100000.

In the BacMam-enabled lantha screen cellular assay the GFP-GSK-3β fusion protein acts as the substrate for the action of $BesO_4$ or LiCl. The Ser-9 phosphorylation of the GSK-3β in the fusion protein is assessed by using a Tb-conjugated anti-pSer9 GSK-3β antibody. The binding of the terbium labeled anti- pSer9GSK-3β antibody to the substrate (fusion protein) brings the terbium close enough to the GFP to support the formation of a successful TR-FRET pair (Time Resolved Fluorescence Energy Resonance Transfer). Terbium and GFP forms an effective TR-FRET pair (David Comley, 2006**)** which has the added advantage of minimizing the background fluorescence interference. In this assay the increase in the Ser-9 phosphorylation of the GFP-GSK-3β fusion protein will in turn lead to the formation of abundant TR-FRET pairs. More the number of successful TR-FRET pairs the higher would be the fluorescence signal and it indicates that in the presence of a specific inhibitor at a particular concentration an increase in the pSer9-GSK-3β is observed.

Flow cytometry

Cells were treated with $BeSO_4$ and LiCl for 24 hr. After the treatment period, cells were collected by trypsinization, washed with PBS and fixed with 4% formal dehyde. Cells were then permeabilized with methanol. Cells were blocked with starting block TBS (prod#37543, thermo scientific), supplemented with halt protease and phosphatase inhibitor cocktail (Pierce cat#78442). Fixed and blocked cells were then incubated with pSer9-GSK-3β rabbit monoclonal antibody (clone D85E12, cat#5558, Cell Signaling Technology) over night at 4℃, followed by FITC conjugated goat anti-rabbit secondary antibody (cat#sc-2012, Santa Cruz Biotechnology) at 0.5 µg/1 x 10^6 cells. Cells were run on a BD FACSCalibur flow cytometer collecting 20,000 events per sample each time. Post data collection the FITC channel fluorescence intensity was analyzed using CellQuest Pro Software.

Microscopy

Nikon Inverted Microscope Eclipse TE2000-U (dia-illuminator 100W) was used to capture bright field images of A172 cells treated with different concentrations of $BeSO_4$ and LiCl.

Data analysis

GraphPad Prism v6 (Mac OS) was used for curve fitting, calculating IC_{50}, performing two-tailed t-test (unpaired) with $P < 0.05$ considered as significant. For data sets involving two or more groups per treatment; the data was analyzed using one-way ANOVA ($P < 0.05$ considered significant) with post Tukey's multiple comparision test.

Results

$BeSO_4$ inhibits the kinase activity of purified recombinant GSK-3β *in vitro*

The Z-Lyte assay, which is a FRET-based kinase assay was used to assess the activity of GSK-3β. Purified recombinant GSK-3β enzyme was used in the assay along with the appropriate assay buffers. A synthetic peptide was used as the substrate, which has the coumarin and fluorescein labels conjugated to its ends. The reaction mix was spiked with different concentrations of $BeSO_4$ (μM) or LiCl (mM). The effect of the inhibitors on the kinase activity of GSK-3β was analyzed by monitoring the FRET signal intensity. The activity of the pure recombinant GSK-3β is directly correlated to the FRET signal. Inhibition of GSK-3β leads to decrease in the FRET signal as well. It is observed that with increasing concentration of Be^{2+} and Li^{+}, the activity of pure recombinant GSK-3β is decreasing (Fig. 2a&2b) and it is translated in the form of lower fluorescence signal. It is also observed that Be^{2+} is a much more potent GSK-3β inhibitor compared to Li^{+}. $BeSO_4$ being effective in micromolar concentrations range compared to the effective range of LiCl which is in millimolars. From the in vitro FRET kinase assay the IC_{50}

values were calculated as follows; $BeSO_4$ IC_{50} = 2 µM, LiCl IC_{50} = 12 mM. The *in vitro* FRET kinase assay demonstrates that Be^{2+} inhibits the activity of purified recombinant GSK-3β enzyme and is much more effective than the other established GSK-3β inhibitor Li^{+}.

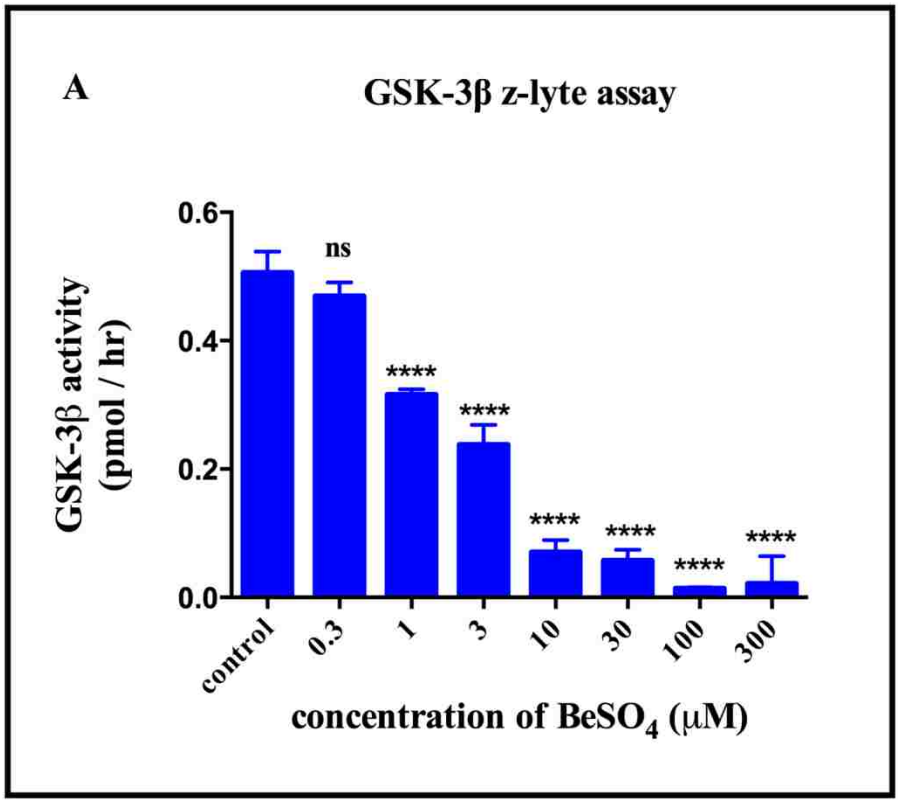


Fig. 2a Effect of beryllium on the in vitro kinase activity of GSK-3β. Pure recombinant GSK-3β enzyme activity was measured using the FRET-based fluorescence assay. The activity of pure recombinant GSK-3β in the absence of inhibitors (control) or in the presence of 0.3, 1, 3, 10, 30, 100, 300 μM $BeSO_4$ was measured. Enzyme activity is measured as formation of the phosphorylated peptide product (mean +/- SD). Data was analyzed using one-way ANOVA ($P <$ 0.05 considered significant compared to control) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. (ns = non significant compared to the control, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$). For Be^{2+} the IC_{50} = 2 μM.

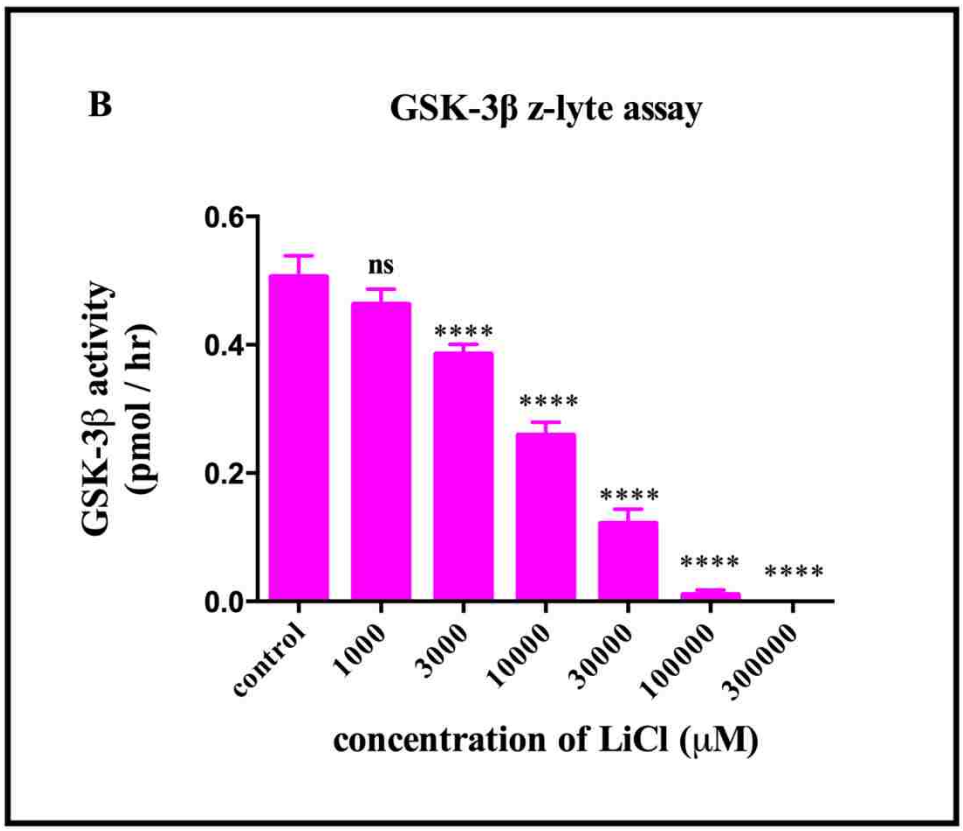


Fig. 2b Effect of lithium on the in vitro kinase activity of GSK-3β. Pure recombinant GSK-3β enzyme activity was measured using the FRET-based fluorescence assay. The activity of pure recombinant GSK-3β in the absence of inhibitors (control) or in the presence of 1, 3, 10, 30, 100, 300 mM LiCl was measured. Enzyme activity is measured as formation of the phosphorylated peptide product (mean +/- SD). Data was analyzed using one-way ANOVA ($P < 0.05$ considered significant compared to control) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant compared to control, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$). For Li^+, IC_{50} = 12 mM.

Specificity of Be^{2+} towards other kinases

To analyze whether Be^{2+} is a specific GSK-3β inhibitor, another kinase i.e. protein kinase A (PKA) was chosen for the FRET-based kinase assay. Li^{+} is a fairly specific GSK-3β inhibitor, which supposedly has no inhibitory effect towards PKA enzyme (Klein and Melton, 1996). In the presence of $BeSO_4$ a decrease in the *in vitro* kinase activity of PKA is observed (Fig. 3a). On the other hand the kinase activity of PKA remains fairly constant in the presence of LiCl at lower concentrations 1-30 mM (Fig. 3b).

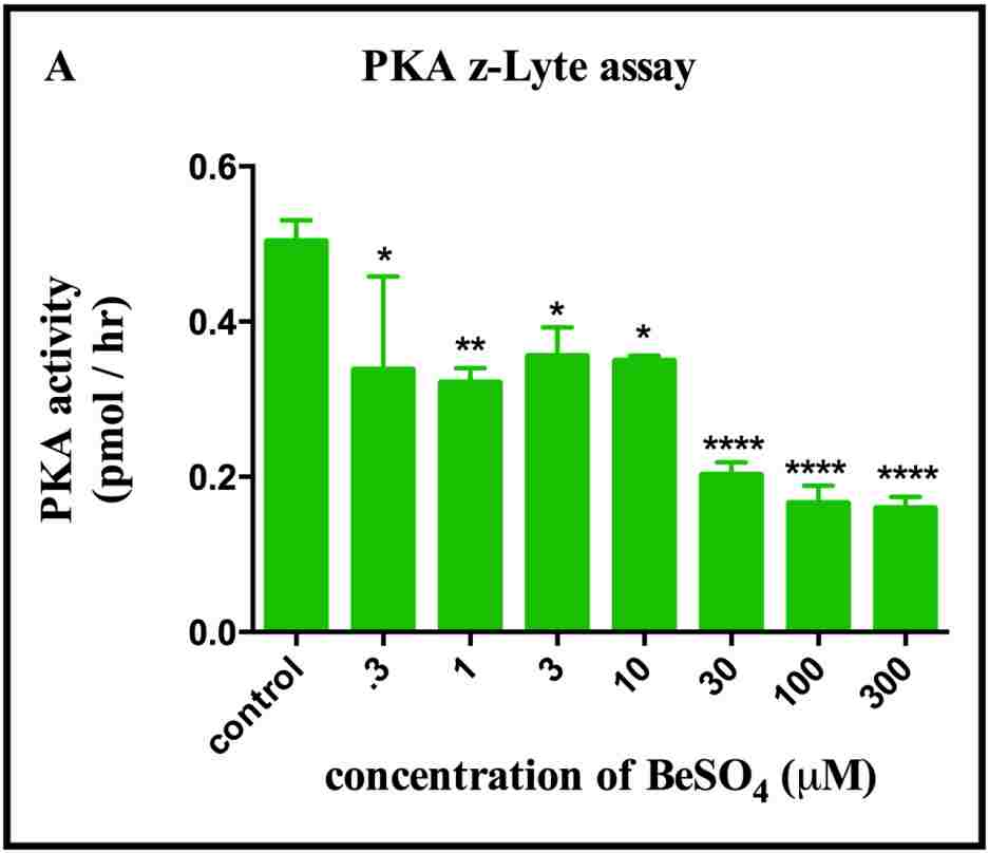


Fig. 3a Effect of Be^{2+} on the in vitro kinase activity of protein kinsae A (PKA). In vitro PKA activity was measured by the FRET-based kinase assay in the absence (control) presence of 0.3, 1, 3, 10, 30, 100 or 300 μM of $BeSO_4$. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significant) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

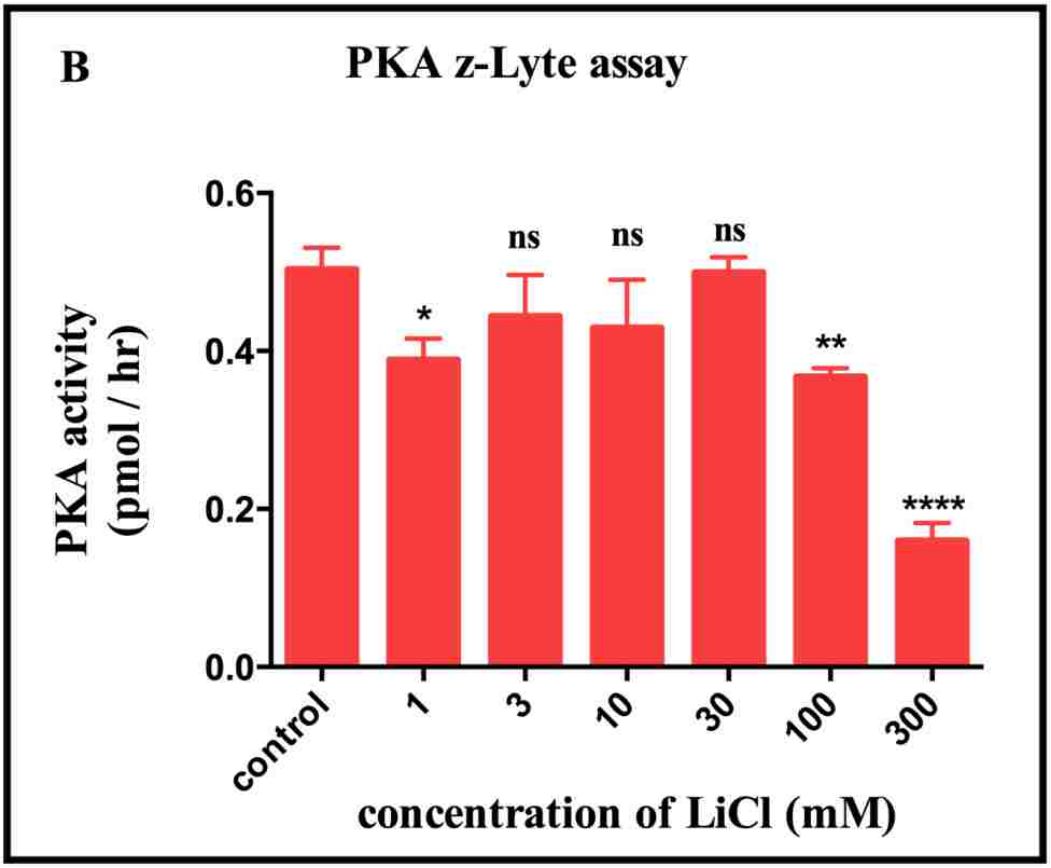


Fig. 3b Effect of Li^+ on the in vitro kinase activity of protein kinsae A (PKA). In vitro PKA activity was measured by the FRET-based kinase assay in the absence (control) presence of 1, 3, 10, 30, 100 or 300 mM of LiCl. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significant) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

Effect of Be^{2+} and Li^{+} on the viability of cells

Use of $BeSO_4$ and LiCl in culture media is bound to produce physiological and morphological effects on mammalian cells especially at higher concentrations. An important observation has been the induction of cellular senescence by $BeSO_4$ at concentrations as low as 10 µM (Gorjala and Gary, 2010) We wished to investigate the effect of $BeSO_4$ and LiCl on the viability and growth of cells. In order to analyze the effect of Be^{2+} - Li^{+} treatment on the viability of cells, A172 cells were cultured in the presence of $BeSO_4$ and LiCl for 24, 48 or 72 hr. The effect of inhibitors on the viability of cells was analyzed using a live cell protease based fluorescence assay. The viability assay shows that beryllium does not have any major impact on the viability of A172 cells even at concentrations higher than its IC_{50} of GSK-3β (Fig. 4). Lithium caused a significant decrease in the number of viable cells at 24 hr; when used at a concentration of 30 mM or more.When treated with 10-100 µM $BesO_4$ for 24 hr no major morphological changes were observed in A172 cells (appendix II, Fig. 2.1). For LiCl treatment, a concentration above 20 mM produced a marked decrease in the number of viable cells (Fig. 4). Morphologically as well A172 cells treated with 20 mM or higher concentrations of LiCl look very unhealthy (appendix II, Fig. 2.2)

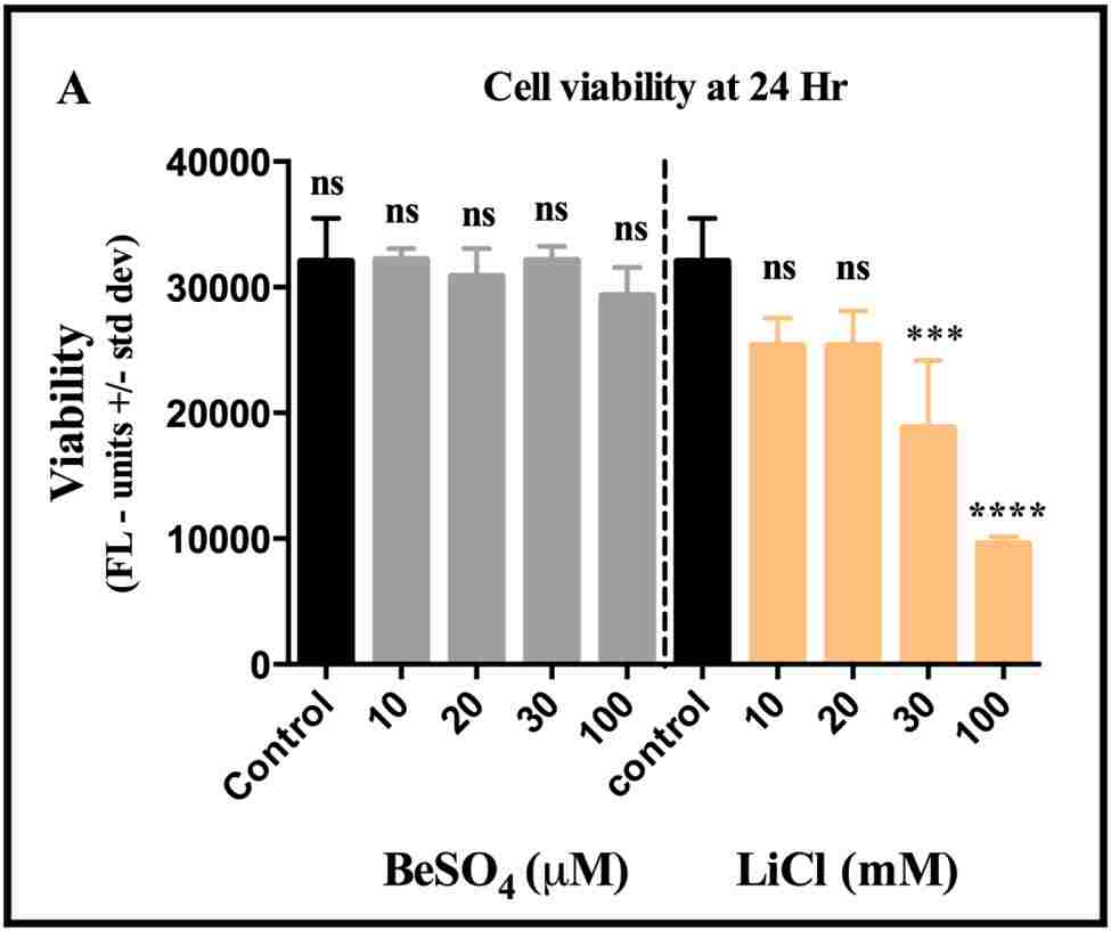


Fig. 4a Effect of $BeSO_4$ and LiCl treatment on cells viability at 24 hr. A172 cells were treated with 0, 10, 20, 30, 100 μM $BeSO_4$ or 10, 20, 30, 100 mM LiCl for 24 hr. Cell viability was assessed using a fluorogenic substrate that can be cleaved by a protease associated with intact viable cells only. Mean intensities (+/- std. dev) are reported here. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significantly different from control) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant compared to the control, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

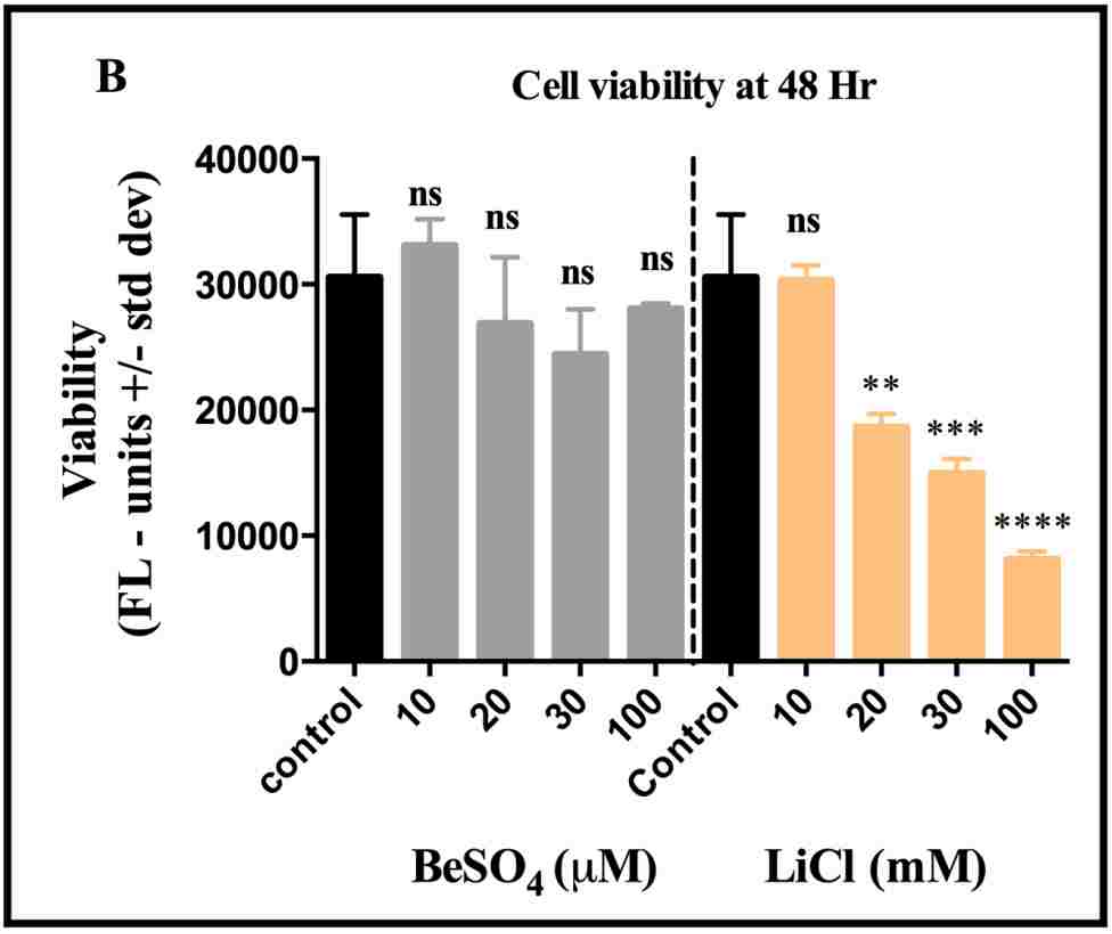


Fig. 4b Effect of $BeSO_4$ and LiCl treatment on cells viability at 48 hr. A172 cells were treated with 0, 10, 20, 30, 100 μM $BeSO_4$ or 10, 20, 30, 100 mM LiCl for 48 hr. Cell viability was assessed using a fluorogenic substrate that can be cleaved by a protease associated with intact viable cells only. Mean intensities (+/- std. dev) are reported here. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significantly different from control) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant compared to the control, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

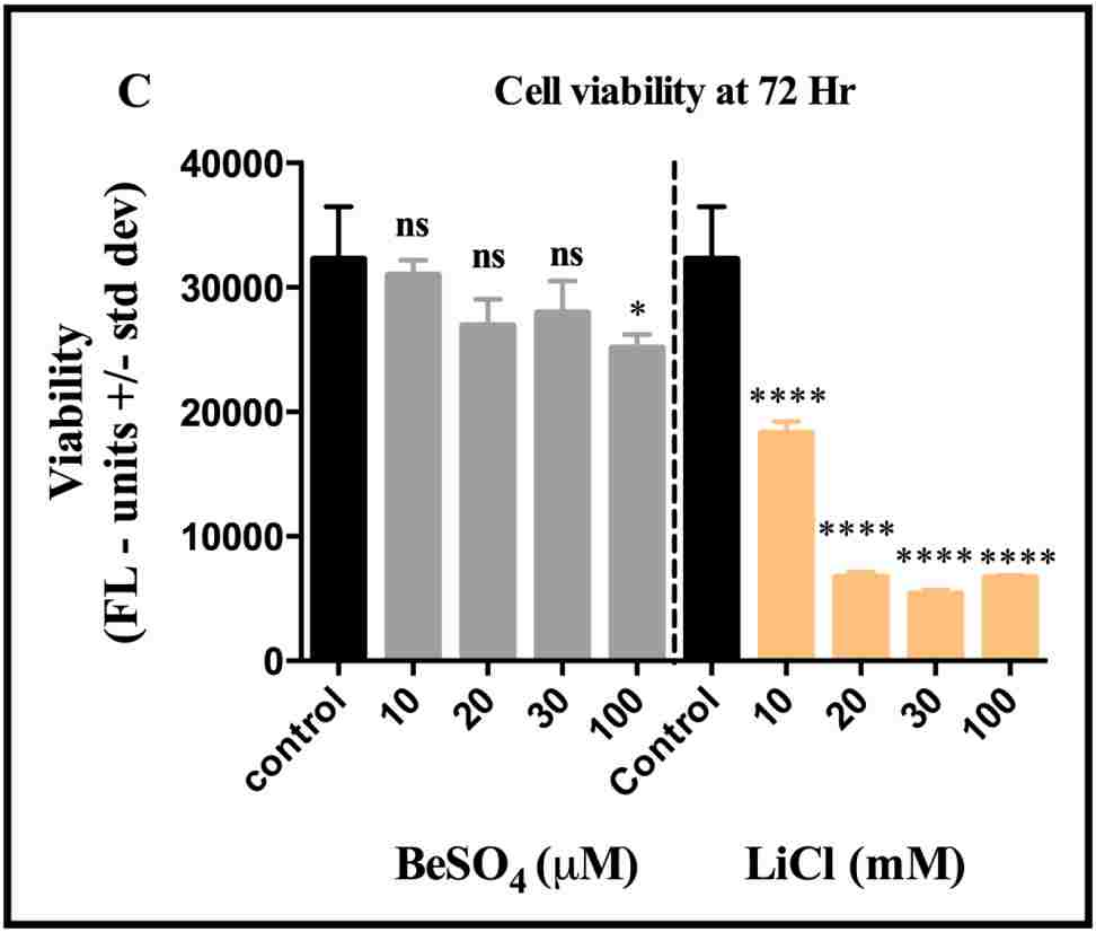


Fig. 4c Effect of $BeSO_4$ and LiCl treatment on cells viability at 72 hr. A172 cells were treated with 0, 10, 20, 30, 100 μM $BeSO_4$ or 10, 20, 30, 100 mM LiCl for 72 hr. Cell viability was assessed using a fluorogenic substrate that can be cleaved by a protease associated with intact viable cells only. Mean intensities (+/- std. dev) are reported here. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significantly different from control) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant compared to the control, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

Beryllium has little effect on the Ser-9 phosphorylation of GSK-3β

Autoregulation of GSK-3β by the inhibitory Ser-9 phosphorylation is an important mechanism by which it is regulated (Frame et al., 2001; Dajani et al., 2001). Lithium treatment leads to an increase in the Ser-9 phosphorylation of GSK-3β (Zhang et al., 2003). In order to investigate the effect of beryllium on the Ser-9 phosphorylation of GSK-3β A172 and HFL cells were cultured in different concentrations of $BeSO_4$ and LiCl for 24 or 48 hr. Phosphorylation of GSK-3β at Ser-9 was assessed by western blotting using a phosphospecific affinity-purified rabbit polyclonal and a phosphospecific mouse monoclonal antibody. Li^+ treatment caused an increase in the Ser-9 phosphorylation of GSK-3β in both A172 (Fig. 5a, b; lane–6, 7, 8) and HFL cells (Fig. 5c, d; lane-6, 7, 8). Fig. 5a, b The Li^+ treatment induced increase in the Ser-9 phosphorylation was observed in the 24 & 48 hr samples. However, Be^{2+} treatment did not elicit any drastic change in the Ser-9 phosphorylation of GSK-3β in A172 (Fig. 5a, b; lane–2, 3,4) or HFL cells (Fig. 5c, d; lane-3, 4, 5) at 24 or 48 hr. To rule out the possibility that the lack of increase in the Ser-9 phosphorylation of GSK-3β is not due to the failure of Be^{2+} treatment, the levels of p53 were assessed in the same samples. Beryllium treatment is known to cause an increase in the levels of p53 (Coates et al., 2007; Gorjala and Gary, 2010). As expected Be^{2+} treatment caused an increase in the level of p53 (Fig. 5a-d), thus the p53 western serves as a positive control for Be^{2+} induced upregulation of protein expression. The total GSK-3α and GSK-3β levels remained constant in the A172 and HFL cells indicating that the Li^+ induced increase in the Ser-9 phosphorylation of GSK-3β is not due to the increase in the total GSK-3β levels (Fig. 5a-d). Actin serves as a loading control (Fig. 5a-d).

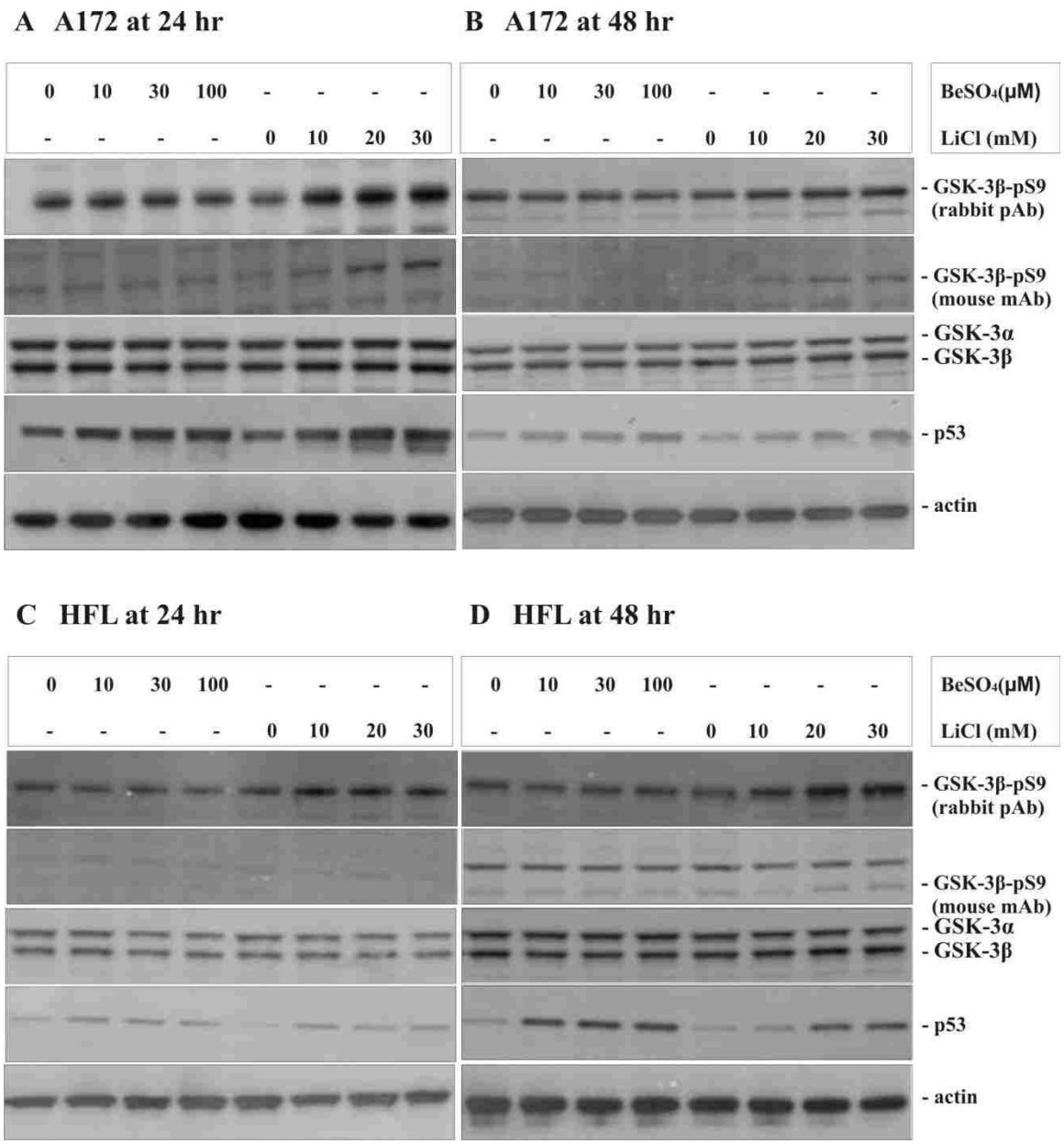


Fig. 5 Li^+ treatment caused an increase in the Ser-9 phosphorylation of GSK-3β and Be^{2+} does not. The protein levels of total GSK-3α/β, GSK-3β phosphorylated at Ser-9 (using two different antibodies), p53 and actin were assessed via western blotting. A172 cells were treated with 0, 10, 30, or 100 μM $BeSO_4$ or 0, 10, 20 or 30 mM LiCl. A172 cells - (**a**) 24 hr or (**b**) 48 hr; HFL cells – (**c**) hr or (**d**) 48 hr.

Quantitative analysis of the Ser-9 phosphorylation of GSK-3β

Li^+ is a monovalent metal cation inhibitor of GSK-3β competing for its Mg^{2+} binding site (Ryves and Harwood, 2001) and also leads to an increase in the inhibitory Ser-9 phosphorylation at physiologically relevant concentrations. Be^{2+} too is a metal cation inhibitor of GSK-3β and we expected it to have similar effect on the Ser-9 phosphorylation of GSK-3β. On the contrary Be^{2+} when used at physiologically relevant concentrations did not induce any appreciable change (increase) in the Ser-9 phosphorylation of GSK-3β as observed in the western blotting experiments (Fig. 5). As an alternate approach a TR-FRET based assay and flow cytometry analysis was used to study the effect of Be^{2+} and Li^+ on the Ser-9 phosphorylation of GSK-3β. The quantification of the Ser-9 phosphorylation signal from GSK-3β was done via flow cytometry. A172 cells were fixed using 4% fomalin solution followed by permeabilization and probed with a flow cytometry specific pSer9-GSK3β antibody. It was observed that Li^+ treatment of A172 cells caused a dose dependent increase in the Ser-9 phosphorylation of GSK-3β. Be^{2+} treatment had little effect on the Ser-9 phosphorylation of GSK-3β in comparision to Li^+ (Fig. 6).

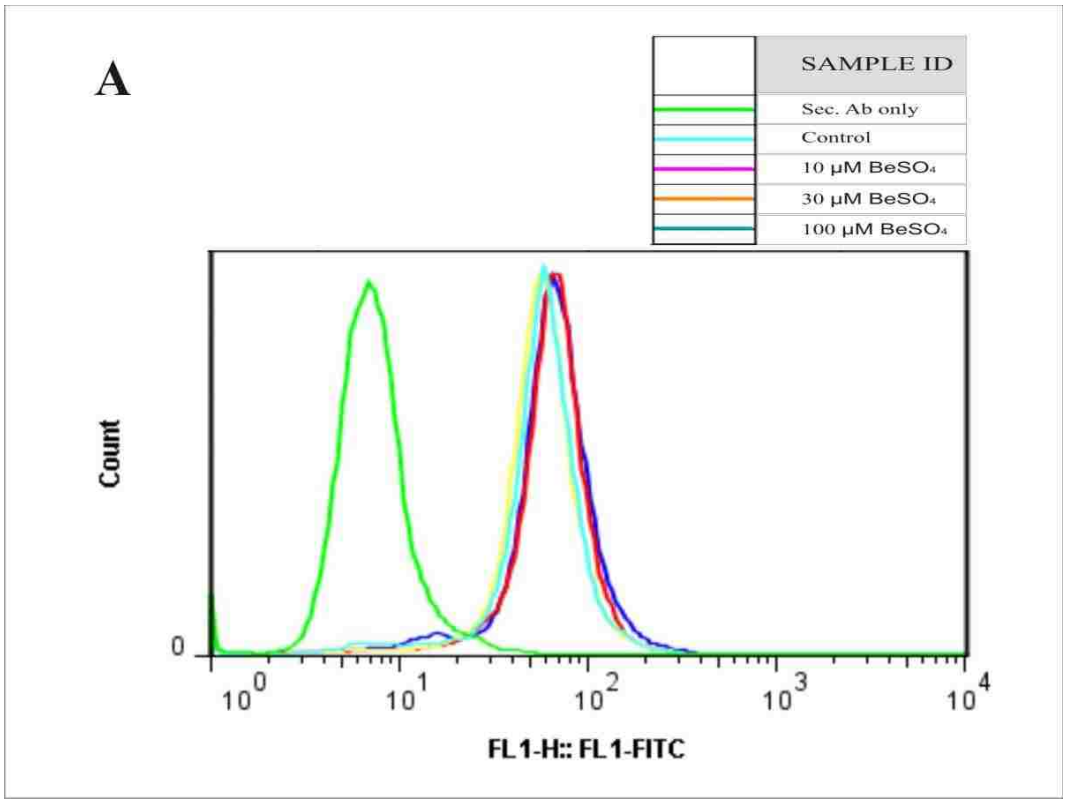


Fig. 6a Analyzing the effect of Be^{2+} treatment on the Ser-9 phosphorylation of GSK-3β using flow cytometry. A172 cells were treated with 0, 10, 30, 100 $BeSO_4$ for 24 hr. The change in the pSer-9 status of GSK-3β was assessed using a pSer9-GSK3β primary antibody and FITC conjugated secondary antibody. Each flow cytometry histograms represents the mean fluorescence per cell value obtained from independent replicates for each inhibitor used at different concentrations.

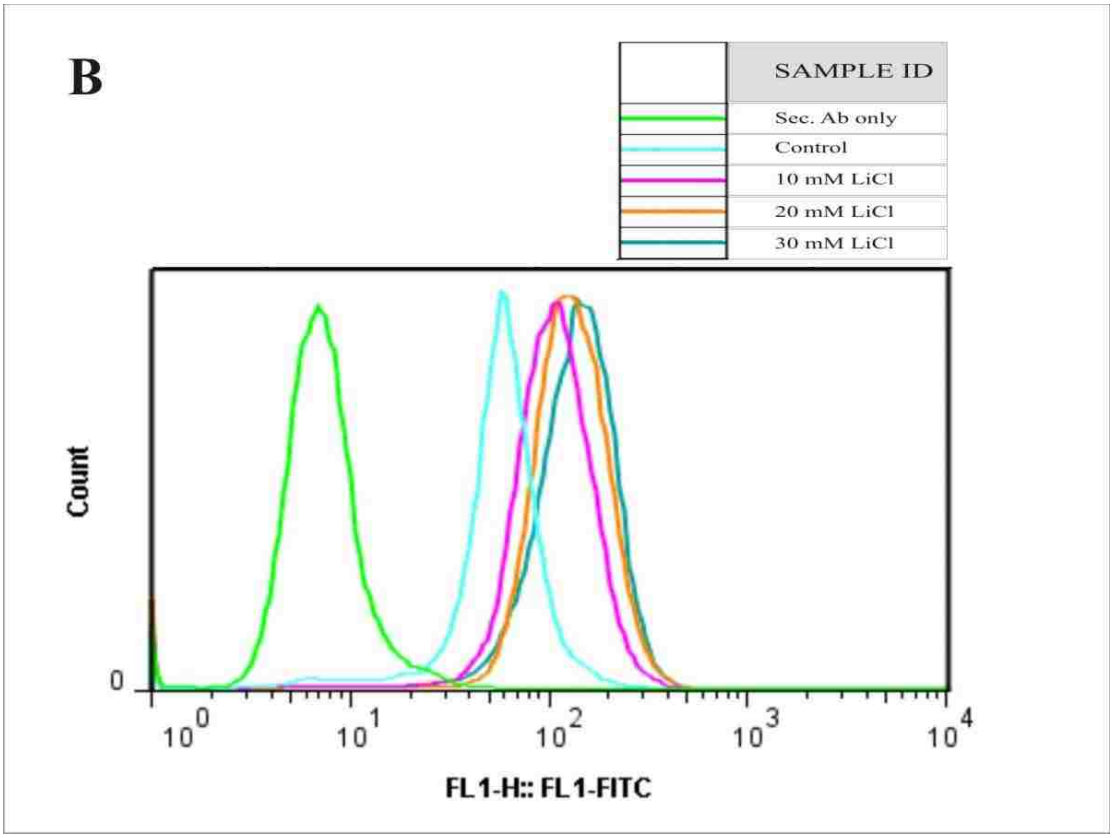


Fig. 6b Analyzing the effect of Li^+ treatment on the Ser-9 phosphorylation of GSK-3β using flow cytometry. A172 cells were treated with 0, 10, 20, 30 mM LiCl for 24 hr. The change in the pSer-9 status of GSK-3β was assessed using a pSer9-GSK3β primary antibody and FITC conjugated secondary antibody. Each flow cytometry histograms represents the mean fluorescence per cell value obtained from independent replicates for each inhibitor used at different concentrations.

Table 6. Analysis of mean fluorescence/cell (Ser-9 phosphorylation) at 24 hr

Treatment	**Duration**	**Mean Fluorescence/Cell (% of control +/- std dev)**
Control	24 hr	100 (+/- 2)
10 µM $BeSO_4$	24 hr	98.9 (+/- 1)
30 µM $BeSO_4$	24 hr	108 (+/- 5)
100 µM $BeSO_4$	24 hr	120 (+/- 8)
10 mM LiCl	24 hr	185 (+/- 12)
20 mM LiCl	24 hr	221 (+/- 8)
30 mM LiCl	24 hr	245 (+/- 25)
Control	48 hr	100 (+/- 2)
10 µM $BeSO_4$	48 hr	102 (+/- 4)
30 µM $BeSO_4$	48 hr	110 (+/- 5)
100 µM $BeSO_4$	48 hr	105(+/- 2)
10 mM LiCl	48 hr	200 (+/- 15)
20 mM LiCl	48 hr	247 (+/- 19)
30 mM LiCl	48 hr	282 (+/- 3)

As a final verification the TR-FRET lantha screen based assay was used to quantify the effect of Be^{2+} and Li^{+} on the Ser-9 phosphorylation of GSK-3β. The BacMam virus based transfection system was used to introduce the GFP-GSK-3β fusion protein into A172 cells. The transfected cells were treated with $BeSO_4$ and LiCl for 24 hr. Cell lysates obtained from the Be^{2+} or Li^{+} treated A172 cells were probed with Tb-conjugated antibody that binds to the GFP-GSK-3β fusion protein phosphorylated at Ser-9. It leads to the formation of a successful FRET pair with Tb serving as the FRET donor and GFP acting as the FRET acceptor. The FRET donor and acceptor are brought together to generate a successful FRET signal because of the binding of Tb-conjugated antibody to the pSer9 of GSK-3β. Increase in the Ser-9 phosphorylation of the GSK-3β is translated in the form of increase in the FRET signal. Be^{2+} has no significant effect on the Ser-9 phosphorylation of GSK-3β (Fig. 7a). Li^{+} on the other hand caused a concentration dependent increase in the Ser-9 phosphorylation of GSK-3β (Fig. 7b).

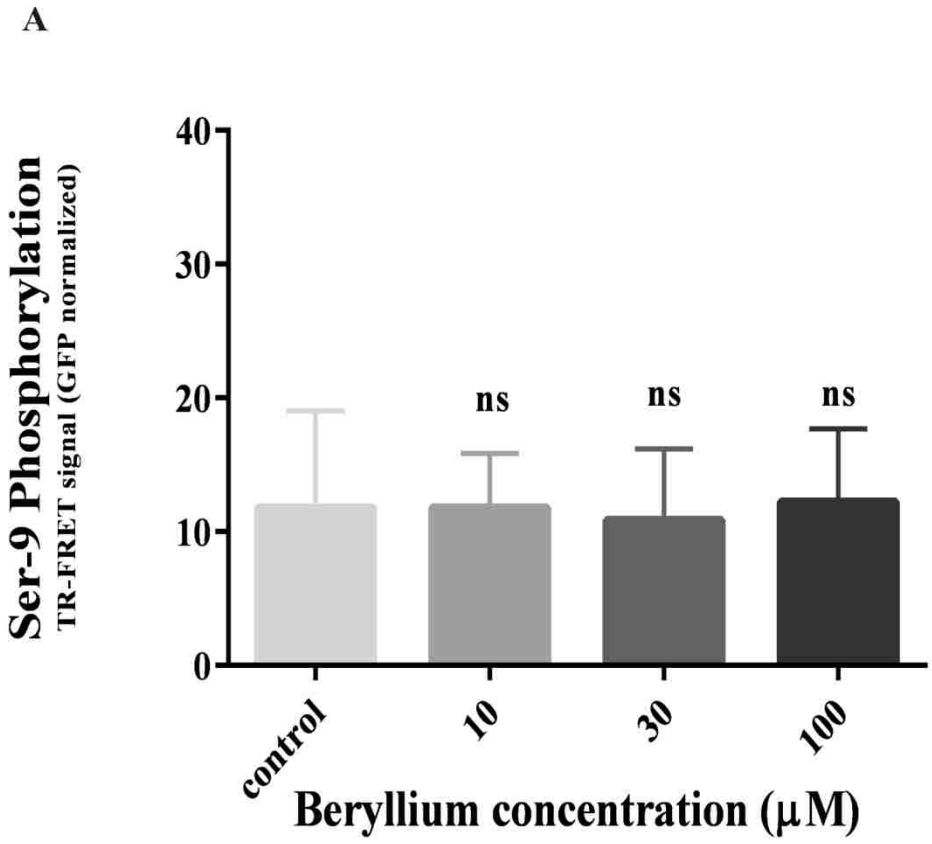


Fig. 7a Be^{2+} treatment has no effect on the Ser-9 phosphorylation of GFP-GSK-3β fusion protein. A172 cells expressing GFP-GSK3β fusion protein were treated with 0, 10, 30 or 100 μM $BeSO_4$ for 24 hr. The Ser-9 phosphorylation of the GFP-GSK-3β was measured using the TR-FRET assay (mean +/- SD). One-way ANOVA with Sidak's multiple comparisons test was used to obtain *P* values comparing the each dosage group with its corresponding control group (*P* <0.05 considered as significant; *ns* - not significant; * - significant).

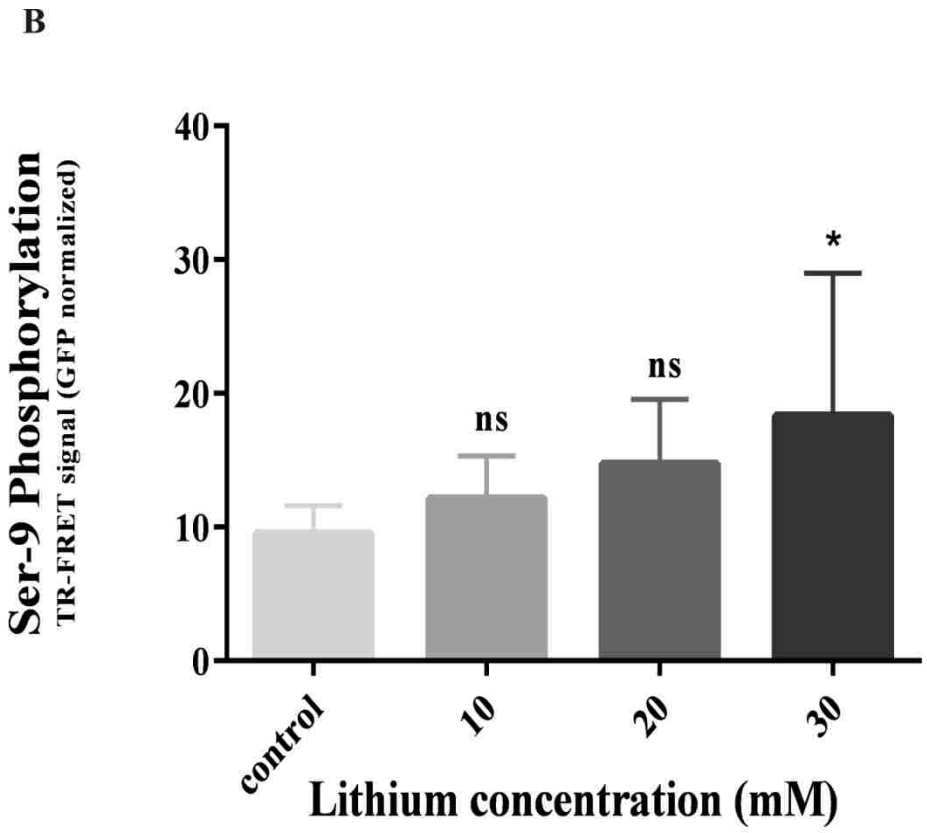


Fig. 7b Li^+ treatment induces an increase in the Ser-9 phosphorylation of GFP-GSK-3β fusion protein. A172 cells expressing GFP-GSK3β fusion protein were treated with 0, 10, 20 or 30 mM LiCl for 24 hr. The Ser-9 phosphorylation of the GFP-GSK-3β was measured using the TR-FRET assay (mean +/- SD). One-way ANOVA with Sidak's multiple comparisons test was used to obtain *P* values comparing the each dosage group with its corresponding control group (*P* <0.05 considered as significant; *ns* - not significant; * - significant).

Discussion

GSK-3β is an important kinase regulating various signaling pathways. GSK-3β plays a critical role in cell divison, cell adhesion and apoptosis (Frame and Cohen, 2001; Doble and Woodgett, 2003; Grimes and Jope, 2001). Many important proteins that are part of the cell cycle regulation apparatus are putative targets of GSK-3β. p53, p21 CIP1, mdm 2, cyclin D1 are some of the important GSK-3β-substrates, which plays an important role in cell cycle regulation and are in turn regulated by GSK-3β (reviewed in Sutherland, 2011). De regulation of GSK-3β has been associated with many diseases (Grimes and Jope, 2001; Henriksen and Dokken, 2006; Smalley and Dale, 1999; Peifer and Polakis, 2000). There is considerable interest towards the development of simple and effective GSK-3β inhibitors for therapeutic purpose and also to understand the precise role of GSK-β in different signaling pathways.

Be^{2+} is a cytostatic agent and its cytostatic effects were documented for the first time in an animal limb regeneration model (Thornton, 1949; Chevremont and Firket, 1951). $BeSO_4$ is known to elicit cellular senescence at concentrations as low as 10 μM (Coates et al., 2007; Gorjala and Gary, 2010). Work from our lab shows that Be^{2+} treatment leads to an increase in the level of p53 and p21 CIP1 (Coates et al., 2007; Gorjala and Gary, 2010; Mudireddy et al., 2014). There is a strong possibility that the cytostatic effects of Be^{2+} could be due to the up regulation of cell cycle regulators like p53 and p21 CIP1. It has been reported that proteins involved in cell cycle regulation such as p53, p21 CIP1, cyclin D1 and mdm2 are important substrates of GSK-3β (reviewed in Sutherland, 2011). It could be possible that the cytostatic effects elicited by beryllium could be partly because of the ability of beryllium to regulate GSK-3β's activity towards its putative substrates (cell cycle regulators). GSK-3β is a Mg^{2+} and ATP dependent enzyme and it has been reported by Ryves et al that Be^{2+} could inhibit GSK-3β enzyme by competing for both the Mg^{2+} and ATP binding sites (Ryves et al., 2002). The available information suggests that beryllium could possibly function as an effective GSK-3β inhibitor.

Historically majority of the research work related to beryllium has been directed towards establishing its propensity to act as a carcinogen. Beryllium at high concentrations and when administered via the nasal route could act as a potential carcinogen (Haley et al., 1990; Nikula et al., 1997; Finch et al., 1998). Moreover beryllium seems to be effective as a carcinogen in rats but not in other mammalian models such as mice and guinea pigs (Schepers, 1961). The question whether Be^{2+} could act as a potential GSK-3β inhibitor was ignored till it was shown that Be^{2+} in the form of $BeCl_2$ can inhibit the in vitro kinase activity of GSK-3β (Ryves et al., 2002). We have used Be^{2+} in the form of $BeSO_4.4H_2O$ and demonstrated that it can inhibit the in vitro kinase activity of the pure recombinant GSK-3β protein (Fig. 2a). In our study we found the IC_{50} of Be^{2+} to be ~ 2 μM, which is quite close to the IC_{50} = 6 μM value reported by Ryves et al. Be^{2+} was found to be a potent GSK-3β inhibitor acting at a much lower concentration compared to Li^+. Be^{2+} was also found to be inhibiting the activity of endogenous GSK-3β activity in A172 and HFL cells (Mudireddy et al., 2014). The ability of Be^{2+} to inhibit the activity of GSK-3β at low concentrations has the added advantage of Be^{2+} being much less toxic to the cells compared to Li^+ at physiologically relevant concentrations (Fig. 4). The low IC_{50} value of Be^{2+} combined by its low cytotoxicity, points to the fact that Be^{2+} could act as a much more potent and effective GSK-3β inhibitor compared to Li^+.

To investigate whether Be^{2+} can inhibit the activity of other related kinases, protein kinase A (PKA) was selected as a negative control. PKA is closely associated with regulation of GSK-3β and is known to phosphorylate GSK-3β at the Ser-9 residue (Fang et al., 2000; Li et al., 2000; Tanji et al., 2002). Hence PKA could serve as a good candidate to investigate the specificity of beryllium towards other kinases. The effect of Be^{2+} on the kinase activity of pure recombinant PKA was analyzed using the FRET- based kinase assay (Fig. 3a). Be^{2+} seems to be inhibiting the in vitro kinase activity of purified recombinant PKA protein. Li^+ supposedly has no effect on the kinase activity of PKA (Fig. 3b) at a concentration that is within the range of its GSK-3β-IC_{50}.

However at high concentrations i.e 100 – 300 mM, Li^+ seems to be inhibiting the in vitro kinase activity of pure recombinant PKA protein (Fig. 3b). To address the specificity of Be^{2+} towards other kinases it is necessary to analyze the effect of Be^{2+} treatment on other closely related kinases. To provide a comprehensive answer whether Be^{2+} is an indiscriminate kinase inhibitor or a specific GSK-3β inhibitor it would be prudent to include as many kinases in this study.

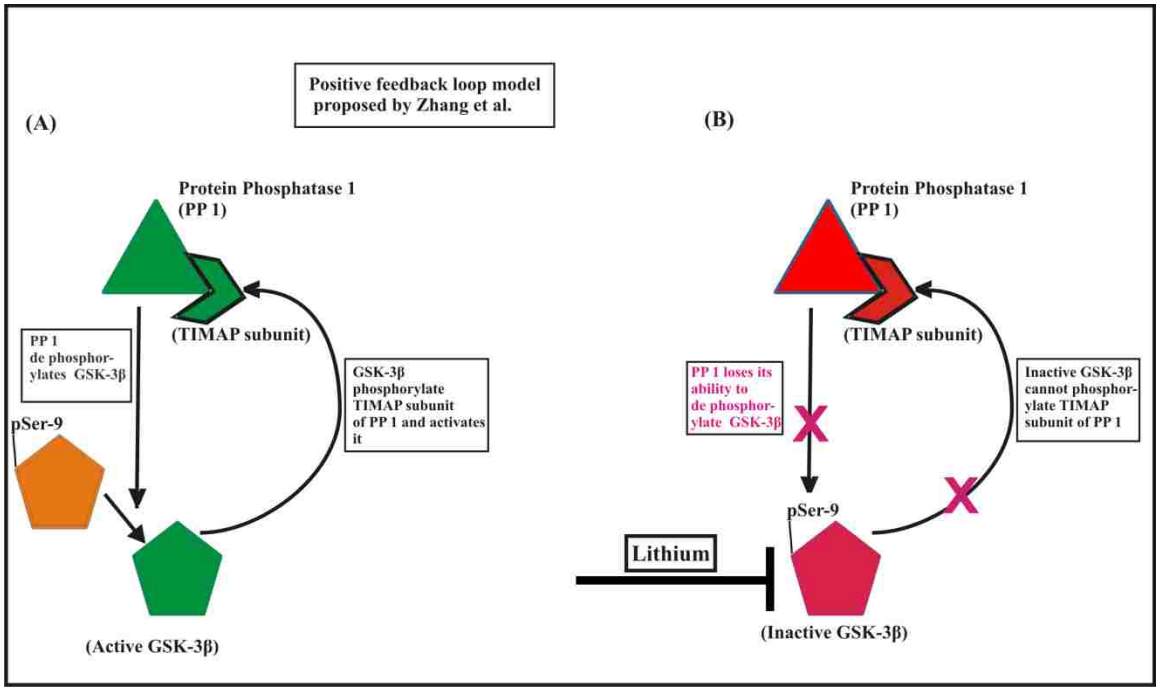
The most surprising aspect of this study is the lack of prominent increase in the Ser-9 phosphorylation of GSK-3β (Fig. 5, 6, 7a). Unlike Li^+, Be^{2+} has minimal effect on the Ser-9 phosphorylation status of GSK-3β. The Ser-9 residue on the N-terminal tail of the GSK-3β protein plays an important role in its regulation. The N-terminal Ser-9 residue when phosphorylated acts a pseudo substrate thus blocking the actual substrate-GSK-3β interaction. The inhibition of GSK-3β induced by the Ser-9 phosphorylation is reversible and the GSK-3β-TIMAP-PP1 feedback loop plays a critical role in it (**refer schematic diagram 3 on page 73**). TIMAP serves as a substrate/target of GSK-3β and is an important regulatory subunit of protein phosphatase 1 (PP1). GSK-3β mediated phosphorylation of TIMAP, up regulates the phosphatase activity of PP1 (Li et al., 2007). The phosphatase activity of PP1 facilitates a decrease in the phosphorylation at Ser-9 of GSK-3β, there by having a positive effect on the GSK-3β-activity. The activation of GSK-3β has an enhancing effect on the activity of PP1 via the GSK-3β mediated TIMAP phosphorylation. Activated PP1 in turn leads to decrease in the pSer-9 of GSK-3β. Hence GSK-3β and PP1 regulate each other via a positive feedback loop. Activated GSK-3β leads to enhanced phosphatase activity of PP1; PP1 in turn works towards decreasing the pSer-9 of GSK-3β. Li^+ inhibits the activity of GSK-3β by inducing Ser-9 phosphorylation, which in turn negatively regulates the phosphatase activity of PP1. The Li^+ induced inhibition of GSK-3β has a compounding effect on the pSer9-GSK-β status because of the perturbation in the GSK-3β-TIMAP-PP1 feedback loop (Zhang et al., 2003). It is quite plausible that the lack of appreciable

increase in the Ser-9 phosphorylation of GSK-3β in the presence of Be^{2+} could be because of its inability to upset the GSK-3β-TIMAP-PP1feedback loop.

However the GSK-3β-TIMAP-PP1feedback loop may not be the only mechanism by which Li^{+} regulates the Ser-9 phosphorylation of GSK-3β. It has been observed that Li^{+} treatment leads to activation and phosphorylation of phosphotidylinositol 3-kinase (PI3K) and the threonine/serine kinase Akt/PKB respectively (Chalecka-Franaszek and Chuang, 1999). PI3K and Akt/PKB are important constituents of the insulin signaling pathway, which ultimately leads to activation of glycogen synthase via the inhibition of GSK-3β. Akt/PKB is known to phosphorylate GSK-3β at Ser-9 and inactivate it (Sutherland et al., 1993; Cross et al., 1994, 1995; Stambolic and Woodgett, 1994). Li^{+} is an insulin mimetic agent (Cheng et al., 1983; Bosch et al., 1986; Woo et al., 2000). It is possible that like insulin, Li^{+} too caused an increase in the Ser-9 phosphorylation of GSK-3β via the activation/phosphorylation of Akt/PKB. However according to some reports Li^{+} has no effect on the phosphorylation of Akt/PKB (De Sarno et al., 2002). Another possible explanation for the minimal increase in the Ser-9 phosphorylation of GSK-3β in response to Be^{2+} could possibly be because of its in ability to interact/affect a critical component of the insulin signaling pathway.

This study adds valuable information to establish Be^{2+} as a metal cation that elicits strong inhibition of GSK-3β and demonstrates that Be^{2+} is a potent and efficient GSK-3β inhibitor compared to Li^{+}.

Schematic diagram 3: GSK-3β-TIMAP-PP1 positive feedback loop model proposed by Zhang et al. (Zhang et al., 2003)



CHAPTER 5

Differential regulation of glycogen synthase and β-catenin by beryllium: a unique inhibitor of GSK-3β

Introduction

Glycogen synthase kinase 3 (GSK-3) is an important serine/threonine kinase found in all eukaryotes. GSK-3 was first isolated in its homogenous form from rabbit skeletal muscle cells (Embi et al., 1980). The name glycogen synthase kinase refers to its ability to phosphorylate glycogen synthase, a key regulatory element of glycogen synthesis pathway. There are two isoforms of mammalian GSK-3: GSK-3α and GSK-3β, which are not functionally interchangeable in spite of sharing 95% sequence identity in their kinase domains (Woodgett, 1990). Functional GSK-3α cannot alleviate the lethality induced in mouse embryos due the homozygous deletions of exon 2 of GSK-3β (Hoeflich et al., 2000).

GSK-3β is a fascinating enzyme playing a central role in extremely diverse intra cellular signaling pathways like Wnt signaling pathway and hedgehog pathway. GSK-3β regulates glycogen synthesis, gene transcription, apoptosis, protein synthesis and cellular differentiation in various cell types (Frame and Cohen, 2001; Doble and Woodgett, 2003; Grimes and Jope, 2001). The moniker glycogen synthase kinase doesn't justify the ability of GSK-3β to phosphorylate various metabolically and structurally important proteins. There is overwhelming evidence that establishes a plethora of different proteins as validated substrates of GSK-3β like tau, β-catenin, cyclin D1, axin, c-jun, c-myc, Heat Shock Factor – 1, BCL-3, CREB, Histone H1.5, mdm2, p21(CIP1), pyruvate dehydrogenase and many more (reviewed in Sutherland, 2011). Since GSK-3β plays a critical role in the regulation and stability of various important proteins, de-regulation of GSK-3β has been associated with many diseases. Hyper phosphorylation of tau protein by

GSK-3β is one of the primary causes for the development of Alzheimer's disease (Hooper et al., 2008). Role of GSK-3β in the development of insulin resistance and type 2 diabetes has been established in various studies (Henriksen and Dokken, 2006).

GSK-3β is an important constituent of the β-catenin destruction complex along with APC (Adenomatous Polyposis Coli), axin, casein kinase 1 (CK1), protein phosphatase 2A (PP2A) and E3-ubiquitin ligase β-TrCP (Zeng et al., 1997; Behrens et al., 1998; Hart et al., 1998, Ikeda et al., 1998; Itoh et al., 1998; Salic et al., 2000; Kikuchi, 1999). The β-catenin destruction complex is involved in regulating β-catenin turn over and GSK-3β plays a central role in it (**refer schematic diagram 2 on page 14**) Wnt activation results in the repression of GSK-3β activity (Bilic et al., 2007; MacDonald et al., 2009; Metcalfe and Bienz, 2011) leading to stabilization and nuclear translocation of β-catenin (Cook et al., 1996; Huber et al., 1996; Willert et al., 1999). In nucleus β-catenin interacts with TCF/LEF proteins to form a complex that regulates the transcription of TCF/LEF target genes (Huber et al., 1996). The inhibition of GSK-3β associated or bound to the components of β-catenin destruction complex has to be tightly regulated because some of the target genes of β-catenin are proto oncogenes (Dale, 1998; Brantjes et al., 2002).

Apart from the Wnt ligands, insulin is another well characterized inhibitor of GSK-3β (Sutherland et al., 1993; Cross et al., 1995; Shaw et al., 1997). Insulin regulates GSK-3β by inducing an increase in the phosphorylation of its N-terminal Ser-9 residue (Sutherland et al., 1993; Welsh and Proud, 1993; Cross et al., 1995). The phosphorylated Ser-9 residue on the N-terminal region of GSK-3β acts as a pseudo substrate thus blocking the access of putative substrates to GSK-3β's catalytic site (Frame et al., 2001; Dajani et al., 2001). The upstream kinases of the insulin signaling pathway especially Akt/PKB (protein kinase B) plays an important role in the insulin induced inhibition of GSK-3β (Cross et al., 1994, 1995; Stambolic and Woodgett, 1994). Akt/PKB has been found to phosphorylate GSK-3β at the N-terminal Ser-9 residue thus inhibiting it (Shaw et al., 1997; Cross et al., 1994, 1995; Stambolic and Woodgett,

1994). There are other inhibitors of GSK-3β that are also known to induce the inhibitory Ser-9 phosphorylation of GSK-3β (discussed later). The available information suggests that phosphorylation of Ser-9 residue on the N-terminal region of GSK-3β plays an important role in its regulation.

Even though Wnt ligands and insulin have been established as negative regulators of GSK-3β their mode of action seems to be different (Ding et al., 2000). Ding et al showed that insulin seems to be inhibiting GSK-3β activity via the inhibitory Ser-9 phosphorylation route and Wnt ligands could not mediate such an affect. The differential regulation of GSK-3β by insulin and Wnt ligands was supported by the observation that Wnt ligands could not induce the Ser-9 phosphorylation of GSK-3β and insulin failed to induce nuclear localization of β-catenin (Ding et al., 2000). This study demonstrates that the Wnt pathway cannot inhibit the GSK-3β-enzyme fraction involved in the insulin signaling and vice versa. The hypothesis that the GSK-3β fraction involved in the insulin signaling pathway is insulated from the inhibitory effect of Wnt ligands and vice versa is contradicted by reports which suggests the possibility of a cross talk between insulin and Wnt signaling pathways (Desbois-Mouthon et al., 2001; Yi et al., 2008).

Apart from insulin and Wnt, another well characterized inhibitor of GSK-3β is FRAT1. It was observed that FRAT1 selectively inhibits GSK-3β mediated phosphorylation of axin and β-catenin (Thomas et al., 1999). Nonetheless FRAT1 did not interfere with the GSK-3β mediated phosphorylation of glycogen synthase (Thomas et al., 1999). It implies that FRAT1 is able to selectively target the Wnt signaling pathway, while the insulin signaling pathway is insulated from FRAT1's inhibitory effects.

The available data suggests that few GSK-3β inhibitors are able to selectively regulate the activity of GSK-3β depending upon the pathway in which it is involved. This information raises the possibility of GSK-3β existing in two different fractions – the "free GSK-3β" associated with

the insulin signaling pathway and the "bound GSK-3β" involved with the β-catenin destruction complex of Wnt signaling (**refer schematic diagram 2 on page 14**). It can be hypothesized that the ability of GSK-3β-inhibitors to inhibit a specific pathway depends on whether they are inhibiting the "free GSK-3β" or the "bound GSK-3β" or both. Alternatively it is also possible that the pathway selectivity of the GSK-3β-inhibitors depends on their effect on the other components of the signaling pathways (apart from GSK-3β). It would be interesting to investigate the effect of GSK-3β-inhibitors on the other members of the insulin signaling pathway such as Akt/PKB or IRS-1. Additionally analyzing the effect of GSK-3β-inhibitors on the proteins involved in the β-catenin destruction complex (axin, APC, PP2A) could provide us with an insight into the underlying mechanism behind the differential regulation of GSK-3β.

As discussed earlier inhibition of GSK-3β activity leads to stabilization and nuclear localization of β-catenin in the nucleus (Cook et al., 1996; Willert et al., 1999; Behrens et al., 1996; Huber et al., 1996). Several studies have directly linked the abnormal accumulation of β-catenin with development of various cancers (Smalley and Dale, 1999; Peifer and Polakis, 2000; Thakur and Mishra, 2013). It can be speculated that de-regulation of the "bound GSK-3β" could be playing an important role in the nuclear localization of β-catenin.

In recent times GSK-3β has emerged as an important therapeutic target because of its role in the etiological development of different abnormalities and diseases. It is important to identify potent inhibitors of GSK-3β in order to characterize and understand the impact of GSK-3β de-regulation. There are various classes of GSK-3β inhibitors and the simplest among them is the group containing metal cations. Lithium (Li^+) is a monovalent metal cation that acts as a potent inhibitor of GSK-3β and is commonly used in the form of LiCl salt (Klein and Melton, 1996; Stambolic et al., 1996). Li^+ inhibits the activity of GSK-3β both directly and indirectly (Jope, 2003). GSK-3β is a Mg^{2+} dependent enzyme and Li^+ inhibits GSK-3β by directly competing with

Mg^{2+} ions for the magnesium binding sites on the protein (Ryves and Harwood, 2001; Ryves et al., 2002).

The post translational modification that leads to an increase in the phosphorylation of Ser-9 on GSK-3β is the indirect mode of action for some GSK-3β inhibitors. Like insulin, Li^{+} too leads to an increase in the inhibitory Ser-9 phosphorylation in a wide variety of cells thereby inhibiting GSK-3β enzyme indirectly (Zhang et al., 2003). From the inhibitory Ser-9 phosphorylation perspective, Li^{+} mimics insulin and has a positive effect on glycogen metabolism (Cheng et al., 1983; Bosch et al., 1986; Choi et al., 2000). In addition Li^{+} also mimics Wnt ligands and activates the Wnt signaling pathway, which in turn causes nuclear localization of β-catenin (Stambolic et al., 1996; Hedgepeth et al., 1997). Li^{+} is a reasonably selective GSK-3β inhibitor but its inhibitory concentration range is in millimolars (Ki = 2 mM) (Klein and Melton, 1996). Beryllium (Be^{2+}) another metal cation acts as an efficient inhibitor of GSK-3β and is 1000 times more potent compared to lithium (Mudireddy et al., 2014).

Beryllium in the form of $BeSO_4$ salt acts as a potent GSK-3β inhibitor and is also tolerated well by mammalian cells at concentrations up to 100 μM (Mudireddy et al., 2014). Apart from the fact that Be^{2+} could be competing for both Mg^{2+} and ATP binding sites on GSK-3β not much is known about the mechanism by which Be^{2+} inhibits the activity of GSK-3β (Ryves et al., 2002). Be^{2+} fails to induce an increase in the Ser-9 phosphorylation of GSK-3β in A172 cells (Mudireddy et. al., 2014), indicating that it might be inhibiting GSK-3β by an unknown mechanism. In this study we investigated whether the lack of increase in Ser-9 phosphorylation of GSK-3β in the beryllium treated cells is a universal phenomenon or a cell type specific event. Our results for the first time demonstrates the intra cellular effects of Be^{2+} on important substrates of GSK-3β like glycogen synthase and β-Catenin. This study validates the efficacy of beryllium as a cell culture reagent for GSK-3β inhibition. Our results also suggests that unlike Li^{+}, which shows

an unrestricted inhibitory action on GSK-3β activity, Be^{2+} could be a more pathway-selective GSK-3β inhibitor.

Materials and methods

Cell culture

A172 (human glioblastoma), U87MG (human glioblastoma/astrocytoma), Caki-1 (human kidney carcinoma) and NIH/3T3 (murine embryo fibroblasts) were obtained from American Type Culture Collection (Manassas, VA). RKO E6 human colon carcinoma, A172 E6 and U87MG E6 cells are RKO, A172 and U87MG transfected with pCMV-E6 plasmid expressing the human papillomavirus 16 E6 gene. A172 E6 cells are established and well characterized clones as described in (Xu et al., 2005). The E6 cell lines were a kind gift of Dr. J. Gregory Cairncross (University of Calgary, Calgary, Alberta, Canada). Cells were grown in RPMI 1640 supplemented with 10% Fetal Bovine Serum, (10% Bovine Growth Serum for NIH/3T3 cells), 25 mM Hepes and 1x antibiotic-antimycotic (Invitrogen-Gibco) at 37℃ in 5% CO_2.

Serum starvation

NIH/3T3 cells were grown to 50-60% confluency in 5% BGS RPMI 1640. Cells were washed with serum-less RPMI 1640 and incubated in 2% BGS RPMI for 24 Hr. Cells were then serum starved for 2 Hr after being washed twice with serum-less RPMI 1640. Cells were then stimulated with 100 nM Insulin (Bovine pancreas insulin, sigma #I0516) for 30-60 min before harvesting the cells.

Salt treatment

$BeSO_4.4H_2O$ (Fluka - lot & filling code 413015/1) was used to prepare a 10 mM stock in nanopure H_2O and sterile filtered. The stock solution was used to prepare the appropriate concentrations of beryllium-RPMI 1640 for dosing cells. A 4M LiCl (sigma#L-8895, lot#22K0184) stock was prepared in nanopure H_2O. 1 M stocks of other salts like $CaCl_2$ (sigma #C-5080, lot #10K0197), $MgCl_2$ (sigma #M-2670, lot #91K0108), NaCl (Amresco#0241-500G, lot #0452C125), KCL (sigma#P-3911, lot#91K0142) and $Na_2SO_4.5H_20$ (#SX0760 E-3) were prepared using nanopure H_2O and sterile filtered.

Cells used for all experiments were obtained from a common pool and seeded in appropriate numbers (usually 1.5 – $2.0*10^6$) to obtain sub confluency (50 – 70%). Cells were grown overnight but not more than 24 Hr before dosing them with freshly prepared RPMI 1640 + inhibitors (beryllium or lithium). The untreated/control cells would receive normal (fresh) RPMI 1640 without any beryllium or lithium.

Small molecule inhibitors of GSK-3β

SB216763 (sc-200646A, lot #D2711) and Rottlerin (sc-3550, lot #K1313) were prepared in DMSO.

Western blotting

Cells were grown in 100 mm CELLSTAR polysterene cell culture dishes (cat#664 160) and harvested by trypsinization. Cells were washed twice with phosphate-buffered saline and total cell lysates were prepared using M-PER (cat#78501, Thermo Scientific) supplemented with protease and phosphatase inhibitors (Halt protease inhibitor cocktail kit, Thermo Scientific cat#78442) and phosphatase inhibitors (sodium fluoride - 20 mM, beta glycerol phosphate - 10 mM, sodium ortho vanadate - 0.1 mM, paranitro phenyl phosphate - 20 mM and 1x EDTA) and the cell suspension was gently vortexed for 10 sec. The lysed cell suspension was incubated on ice for 10 min and followed by gentle vortexing for 5 sec and centrifuged at 14,000 RPM for 30

min. Appropriate amount of cell supernatant was added to 5x laemmli SDS sample buffer and boiled immediately for 5 min. Total protein concentration in the supernatant was measured by using BCA assay (Thermo Scientific #23227). Normalized protein samples were loaded onto 8% SDS-PAGE gels for probing with total glycogen synthase, β-catenin and phospho-β-catenin proteins. For probing p53, actin, GSK-3β (Ser-9), total GSK-3β, p53 (1C12), total GSK-3α/β, α-Tubulin, lamin-B and lamin A/C 10% SDS-PAGE gels were used. For the phospho glycogen synthase samples 4-12% bis-tris gradient gels (ref#WG1402BX10 from Novex-Life Technologies) were used. After electrophoresis the proteins were transferred to polyvinylidine difluoride membrane (Millipore cat#IPFL20200, Biorad cat#162-0255). Post transfer the membranes were blocked in 10% milk or starting block TBS (Prod#37543, Thermo Scientific) when probing with phospho antibodies. Primary antibody labeled blots were incubated with the respective HRP-conjugated secondary antibodies and developed with ECL-Plus (GE Healthcare Life Sciences) or clarity western ECL substrate (Bio-Rad cat#170-5061).

Fractionation of cytoplasmic and nuclei proteins

For the preparation of cytoplasmic and nuclear extracts from mammalian cells (A172, U87MG, Caki-1, NIH/3T3, RKO E6, A172 E6 and U87MG E6), NE-PER nuclei and cytoplasmic extraction kit from thermo scientific was used (#78833, Thermo Scientific).

- Cells were grown as explained in the cell culture section above.
- To analyze the effect of beryllium on different cell lines, they were grown in RPMI 1640 supplemented with appropriate concentration of $BeSO_4$ (10 – 100 μM).
- Cells were harvested by trypsinization. Phosphate buffered saline was used to wash the cells twice to remove traces of serum and trypsin. (soyabean trypsin inhibitor was used to nullify the adverse effects of trypsin on the cells).
- 250 μL of CER-I buffer was added to each dry cell pellet and vortexed vigorously for 15 sec.
- The cell suspension in CER-I buffer was incubated on ice for 20 min.

- 13.75 µL of CER-II buffer was added to the cell suspension and vortexed vigorously for 15 sec followed by incubation on ice for 10 min.
- Cell suspension was vortexed again vigorously for 5 sec. Followed by centrifugation at 14,000 RPM for 30 min.
- After centrifugation the supernatant (cytoplasmic proteins) was removed and added to a pre chilled 1.5 ml tube.
- Extra supernatant present on top of the nuclei pellet was discarded. Nuclei pellets were then washed with 400 µL of phosphate buffered saline (to prevent any cross contamination of nuclear fraction with cytoplasmic proteins).
- 110 µL of NER buffer was added to each pellet. Nuclei suspension was mixed well.
- The nuclei suspension was vortexed vigorously for 15 sec followed by incubation on ice for 15 min.
- Nuclei suspension was subjected to 4 cycles of vigorous vortexing followed by 15 min of incubation on ice.
- The nuclei suspension was then centrifuged at 14,000 RPM for 30 min.
- The supernatant containing the nuclear proteins was added to pre chilled 1.5 ml tubes.
- Appropriate amount of cytoplasmic and nuclear supernatants were mixed with 5x laemmli's buffer and boiled for 5 min.

Note: All the steps were carried out at 4˚C.

Total protein concentration in the supernatant was measured by using BCA assay (Thermo Scientific # 23227). Normalized protein samples were loaded onto SDS-PAGE gels for probing.

Antibodies

p53 DO1: Mouse monoclonal IgG_{2b} (cat#sc-126, Santa Cruz Biotechnology, inc) and goat anti-mouse IgG peroxidase (cat#A9917 Sigma Aldrich)

Actin: Goat monoclonal IgG_{2b} (cat#sc-1615, Santa Cruz Biotechnology, inc) and bovine anti-goat IgG-HRP (cat# SC-2350 Santa Cruz Biotechnology, inc)

phospho GSK-3β (Ser-9): Rabbit antibody (#9336S, Cell Signaling Technology) and anti-rabbit IgG-HRP (cat#31460 Thermo Scientific)

phospho GSK-3β D85E12 (Ser-9): Rabbit antibody(#5558, Cell Signaling Technology) and goat anti-rabbit IgG- FITC (cat#2012, Santa Cruz Biotechnology, inc) (for nuclei flow experiment)

Total GSK-3β (H-76): Rabbit polyclonal IgG_{2b} (cat#sc-9166, Santa Cruz Biotechnology, inc) and anti-rabbit IgG-HRP (cat#31460, Thermo Scientific)

Total GSK-3α/β (0011-A): Mouse monoclonal IgG_{2b} (cat#sc-7291, Santa Cruz Biotechnology, inc) and goat anti-mouse IgG peroxidase (cat#A9917 Sigma Aldrich)

β-catenin (E5): Mouse monoclonal IgG1 (cat#sc-7963, Santa Cruz Biotechnology, inc) and goat anti-mouse IgG peroxidase (cat#A9917 Sigma Aldrich)

Phospho β-catenin (Ser-33) – R: Rabbit polyclonal IgG (cat#sc-16743-R, Santa Cruz Biotechnology, inc) and anti-rabbit IgG-HRP (cat#31460, Thermo Scientific)

α -Tubulin (B-7): Mouse monoclonal IgG_{2a} (cat#sc-5286, Santa Cruz Biotechnology, inc) and goat anti-mouse IgG peroxidase (cat#A9917, Sigma Aldrich)

Lamin – B (C12): Mouse monoclonal IgG_{2b} (cat#sc-365214, Santa Cruz Biotechnology, inc) and goat anti-mouse IgG peroxidase (cat#A9917, Sigma Aldrich)

p53(1C12): Mouse monoclonal IgG_1(#2524, Cell Signaling Technology) goat anti-mouse IgG peroxidase (cat#A9917, Sigma Aldrich) (for mouse cell lines)

Lamin A/C (4C11): Mouse monoclonal IgG_{2a} (#4777S, Cell Signaling Technology) goat anti-mouse IgG peroxidase (cat#A9917, Sigma Aldrich)

α-Tubulin: Rabbit polyclonal (#2144S, Cell Signaling Technology) and anti-rabbit IgG-HRP (cat#31460, Thermo Scientific)

phospho-Glycogen synthase (Ser641/Ser645): Rabbit polyclonal (#07-817, EMD Millipore) and anti-rabbit IgG-HRP (cat#31460, Thermo Scientific)

Total Glycogen synthase: Rabbit polyclonal (#3893S, Cell Signaling Technology) and anti-rabbit IgG-HRP (cat#31460, Thermo Scientific)

Lamin-B (C-20): Goat polyclonal IgG (cat#sc-6216, Santa Cruz Biotechnology, inc) (used mostly for nuclei flow experiments)

Secondary antibodies for flow cytometry and immunoflorescence experiments

Anti Mouse IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor 647 Conjugate: (#4410, Cell Signaling Technology)

Donkey anti goat IgG-FITC (cat#sc-2024, Santa Cruz Biotechnology, inc)

Stable nuclei isolation from mammalian cells for flow analysis

A minimum of $5x10^6$ cells are required for stable nuclei isolation.

- A172 Cells were grown in 100 mm dishes and harvested by trypsinization.
- Post trypsinization cells were washed twice with phosphate buffered saline (soyabean trypsin inhibitor was used to nullify the adverse effects of trypsin on the cells).
- Washed cells were resuspended in 500µL of cold nuclei extraction buffer (320mM sucrose, 5mM $MgCl_2$, 10mM HEPES, 2% Triton X-100 at pH 7.4) at approximately 1 mL/1 million cells.

- Cells were vortexed gently for 10 sec and incubated on ice for 10 min.
- Cell suspension was centrifuged at 3,500 RPM for 20 min.
- Isolated nuclei were washed twice with nuclei wash buffer (320mM sucrose, 5mM $MgCl_2$, 10 mM HEPES at pH 7.4, no Triton X-100).
- Isolated nuclei were resuspended in 100 µL of primary antibody solution. Antibody incubation buffer (320mM sucrose, 5mM $MgCl_2$, 10mM HEPES, 1% BSA and 0.1% sodium azide at pH 7.4, no Triton X-100). Recommended primary antibody concentration is 1 µg/ nuclei pellet.
- Isolated nuclei were incubated with the primary antibody solution @ 4˚C overnight (isolated nuclei can remain stable for 24 Hr in the nuclei wash buffer).
- Post primary antibody incubation, the nuclei were washed twice with nuclei wash buffer.
- Nuclei were resuspended in 100 µL of secondary antibody (FITC or Alexa 647 tag) solution at a concentration of 0.5 – 0.75µg/nuclei pellet and incubated at room temperature for 2 Hr (post secondary antibody steps under dark conditions).
- Post secondary antibody incubation the nuclei were washed twice with nuclei wash buffer.
- Nuclei were resuspended in 500 µL of nuclei wash buffer in flow cytometry tubes for analysis.

Immunofluorescence Microscopy

Cells were grown in Matek glass bottom dishes - cover slip # 1.5 (thickness – 0.16-0.19 mm). After the treatment with inhibitors cells were washed twice with phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cells were fixed with freshly prepared 4% paraformaldehyde – PBS (Ca^{2+}&Mg^{2+}) at 37℃ for 10 min. Fixed cells were washed twice with PBS (Ca^{2+}&Mg^{2+}) and permeabilized with 0.2% $Tween_{20}$ - PBS (Ca^{2+}&Mg^{2+}) at room temperature for 15 min. Cells were blocked in antibody incubation buffer (1% BSA + 0.2% $Tween_{20}$ - PBS (Ca^{2+}&Mg^{2+}) for 30 min at room temperature. Cells were incubated in β-catenin

(E5): Mouse monoclonal IgG1 (cat#sc-7963 Santa Cruz Biotechnology, inc) and Lamin-B (C-20): Goat polyclonal IgG (cat#sc-6216 Santa Cruz Biotechnology, inc) overnight at 4˚ C. Secondary antibodies used were anti mouse IgG (H+L), $F(ab')_2$ fragment (Alexa Fluor 647 conjugate: (#4410, Cell Signaling Technology), donkey anti goat IgG-FITC (cat#sc-2024 Santa Cruz Biotechnology, inc) and Hoechst dye to stain chromatin (1 µg/ml, Sigma-Aldrich). Images were collected using Nikon A1R BX40 confocal laser scanning microscopy system. The images were processed using ImageJ.

p53 knock down using stealth RNAi in A172 cells

TP53 validated stealth RNAi (Cat No. 45-1492) from Invitrogen was used to knock down p53 expression transiently in A172 cells.$1.2x10^6$ A172 cells were seeded in 100 mm dishes to obtain 50 – 60% confluency after 12 Hr. The 10% FBS RPMI 1640 was removed and cells were washed once with phosphate-buffered saline, later OptiMem reduced serum media (#11058, Gibco) was added to each dish. 20 nM of the Stealth p53 RNAi oligo II duplex was used since it was less toxic to cells compared to the RNAi Oligo I Duplex. Stealth p53 RNAi oligo II duplex and lipofectamine 2000 (ref #11668-030, Invitrogen/Life Technologies) were diluted in OptiMem individually in such a way that the final volume is equal to 250 µL. The lipofectamine 2000 + OptiMem mix was incubated at room temperature for 5 min. The Stealth p53 RNAi oligo duplex II + OptiMem solution was mixed with lipofectamine 2000 + OptiMem so that the final ratio between the oligo and lipofectamine 2000 is 1:5 (5 µL of Oligo and 25 µL of lipofectamine 2000. The Oligo + Lipofectamine 2000 + OptiMem mix (transfection mix) was incubated at room temperature for 20 min. The Oligo + Lipofectamine 2000 + OptiMem mix was added to A172 cells growing in the OptiMem media. The dishes were incubated at 37˚C for 8 Hr 30 min. The OptiMem media containing the transfection mix was replaced with normal RPMI 1640 (10% FBS + 1% PSF) and RPMI 1640 supplemented with 100 µM $BeSO_4$ or 20 mM LiCl after the 8.5 Hr

incubation time. Cells were harvested (either total cell lysates or cytoplasmic and nuclear fractionations) after culturing them under the influence of beryllium and lithium for 24 Hr.

p53 knock down using shRNA(h) lenti viral particles in A172 cells

Integration of specific short hairpin RNA (shRNA) constructs into genomic DNA of A172 cells can lead to the generation of A172 clones with constitutively down regulated p53 expression. Stable A172 p53 KO or control A172 cell lines were generated by Dr. Priyatham Gorjala using a lentiviral shRNA expression vector. Detailed methods describing the production of these cells are available in Dr. Gorjala's dissertation (Gorjala, 2012).

Addgene clone transfection

The following addgene plasmids were obtained from addgene plasmid repository plasmid#14753–HA GSK-3β wt pcDNA3, plasmid#14754–HA GSK-3β S9A pcDNA3, plasmid#14755–HA GSK-3β K85A pcDNA3. A172 cells were transfected with the above mentioned plasmids to generate stable clones over expressing wt GSK-3β and its mutants. A172 cells were grown to sub confluence in a 24 well plate. 1 µg of plasmid DNA was diluted in 25 µL of plain RPMI 1640 lacking serum and antibiotics. 4 µL of Plus™ was added to the plasmid mix and incubated at room temperature for 15 min. 1 µL of Lipofectamine™ reagent was diluted in 25 µL of plain RPMI 1640 lacking serum and antibiotics. The pre complexed plasmid DNA was mixed with diluted Lipofectamine™ and incubated at room temperature for 15 min. The cells in the 24 well plate was supplemented with 200 µL of serum less RPMI 1640 containing plasmid DNA + Plus™ + Lipofectamine™ reagent and incubated at 37℃ for 6 Hr. The RPMI containing the transfection mix was replaced with normal RPMI 1640 (10% FBS + 1% PSF) and cells were grown for 24 Hr at 37˚C (cells were grown in normal RPMI initially to provide cells with proper growth conditions). Transfected A172 cells were grown in RPMI 1640 supplemented with 400 µg/ml of G418 (cat#1811-23, lot#1213491, Gibco) for first 48 Hr followed by a lower

concentration of 200 µg/ml with G418 acting as selective pressure for isolating and propagating transfection positive A172 cells. Single cell colonies of transfectants were selected via serial dilution in a 96 well plate. The obtained clones were tested for the expression of the respective proteins by analyzing the presence of the HA tag in the lysate samples via western blotting.

Results

A cell type specific event: Be^{2+} induces either an increase or no change in the Ser-9 phosphorylation of GSK-3β depending on the cell type

A. Beryllium does not cause an increase in the Ser-9 phosphorylation of GSK-3β in A172 (Human glioblastoma) and U87MG cells (Human glioblastoma astrocytoma)

Increase in the Ser-9 phosphorylation of GSK-3β is one of the hallmarks of Li^+ induced inhibition of GSK-3β (Chalecka-Franaszek and Chuang 1999; Lochhead et al., 2001; Zhang et al., 2003). We have shown previously that beryllium unlike lithium does not lead to substantial increase in Ser-9 phosphorylation of GSK-3β in A172 and HFL cells (Mudireddy et al., 2014). This was an unexpected result, so we wished to extend this analysis to include additional cell types to see whether the initial observation was a general result or whether it might be cell-type-specific. The Ser-9 phosphorylation status of GSK-3β in A172 and U87MG cells was analyzed using western blotting (#9336S rabbit polyclonal anyibody, Cell Signaling inc.) (Fig. 8) Li^+ served as a positive control. In A172, Li^+ treatment (20 mM) caused an increase in the Ser-9 phosphorylation of GSK-3β at 24 hr, an observation that is consistent with our prior results (Mudireddy et al., 2014). Treating A172 with increasing concentrations of Be^{2+} did not elicit any appreciable change in the Ser-9 phosphorylation of GSK-3β. Treating U87MG cells with Be^{2+} (100 µM) at 0, 6, 12 and 24 hr did not induce any increase in the Ser-9 phosphorylation of GSK-3β or total GSK-3β (sc#9166, Santa Cruz Biotechnology, inc) (Fig. 9a). U87MG cells when

treated with Be^{2+} (10, 100 µM) or Li^{+} (20 mM) at 24 hr in an independent experiment did not cause any increase in the Ser-9 phosphorylation of GSK-3β (Fig. 9b).

An established effect of Be^{2+} treatment at molecular level is the increase of p53 protein levels in mammalian cells (Lehnert et al., 2001; Coates et al., 2007; Gorjala and Gary, 2010). The increase in the p53 protein level in the A172 (Fig. 8) and U87MG (Fig. 9a&9b) validates the effect of Be^{2+} treatment on the mentioned cell lines. Thus, the p53 westerns serve as a positive control for Be^{2+}- induced change in protein expression, which strengthens the conclusion that Be^{2+} does not cause any substantial increase in the Ser-9 phosphorylation of GSK-3β in A172 and U87MG cells at 24 hr.

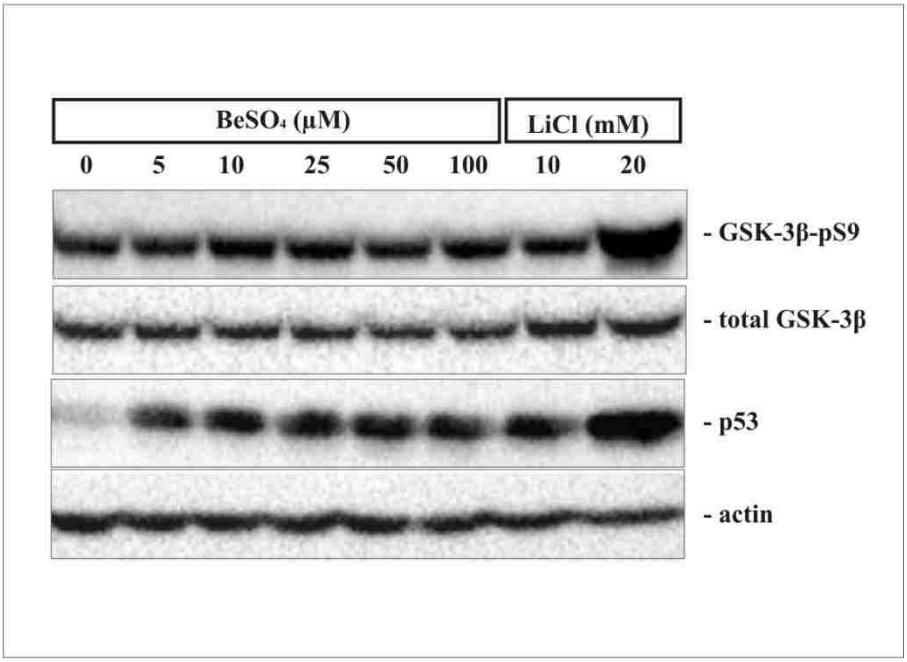


Fig. 8 Beryllium does not cause induction of GSK-3β Ser-9 phosphorylation in A172 cells at 24 hr. A172 cells were treated with 0, 5, 10, 25, 50 or 100 μM $BeSO_4$ or 10 or 20 mM LiCl for 24 hr. Ser-9 phosphorylation of GSK-3β (**GSK-3β-pS9)** was detected by western blotting using phospho-GSK-3β (Ser-9) antibody. Total GSK-3β and actin protein levels are shown as loading control. Li^+ treatment caused a prominent increase in the Ser-9 phosphorylation of GSK-3β but not in Be^{2+} treated A172 cells.

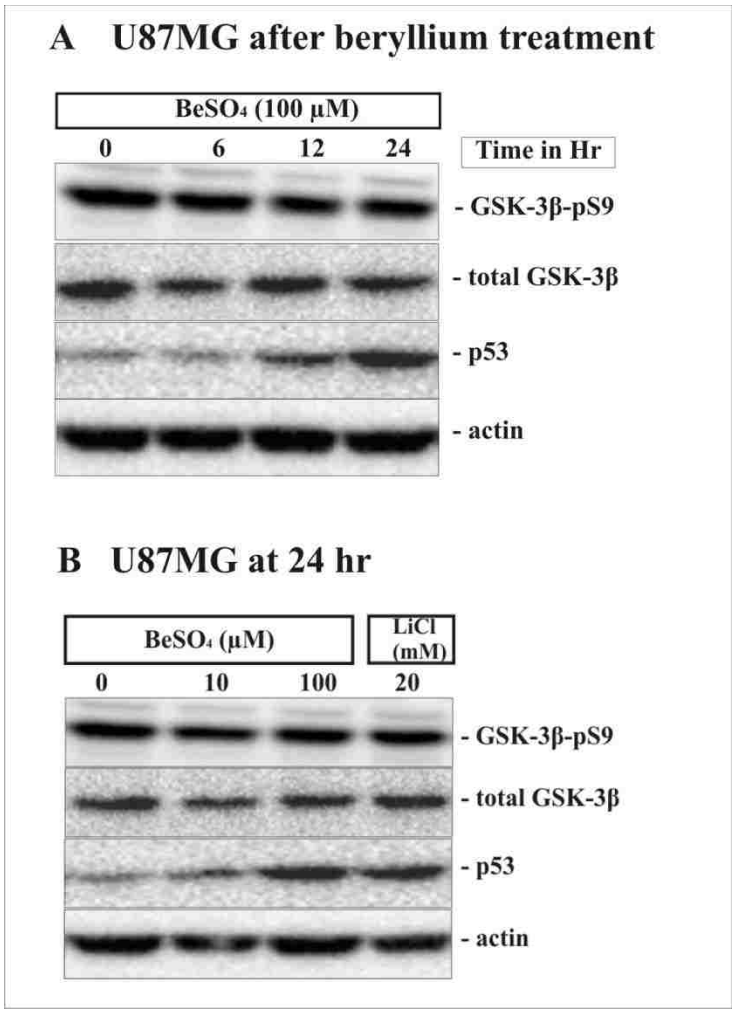


Fig. 9 Beryllium does not cause induction of GSK-3β Ser-9 phosphorylation in U87MG cells. a U87MG cells treated with 100 µM $BeSO_4$ did not show any increase in the Ser-9 phosphorylation of GSK-3β at 0, 6, 12 or 24 hr. **b** U87MG cells treated with 0 or 20 mM LiCl or 10 or 100 µM $BeSO_4$ in an independent experiment again show the lack of increase in the Ser-9 phosphorylation of GSK-3β in the presence of Be^{2+}.

B. Beryllium induces an increase in the Ser-9 phosphorylation of GSK-3β in NIH/3T3 (murine embryo fibroblast) and Caki-1(human kidney carcinoma) cells

To analyze, whether the inability of Be^{2+} to induce Ser-9 phosphorylation of GSK-3β is a cell type specific event or a universal phenomenon, two additional cell systems were included in this study. The Ser-9 phosphorylation status of GSK-3β in these cell lines was investigated post Be^{2+} treatment.

To investigate the effect of Be^{2+} on the Ser-9 phosphorylation of GSK-3β in NIH/3T3 cells (murine fibroblasts), cells were treated with increasing concentration of $BeSO_4$ for 24 hr. A prominent increase in the Ser-9 phosphorylation of GSK-3β was observed in the Be^{2+} treated NIH/3T3 cells (Fig. 10a). The p53 westerns demonstrate that 25-100 μM $BeSO_4$ represents a sufficient dose to effect physiological change in this cell type. The p53 (1C12) antibody used here is specific for mouse cell lines and produces much weaker bands compared to the p53 DO1 (sc#126, Santa Cruz Biotechnology, Inc) used for human cell lines. In our study the p53 DO1 antibody did not yield any visible p53 bands in mouse samples hence the p53 (1C12) mouse specific antibody was used, even though it produces weak bands. However the p53 (1C12) results confirmed the expected results in mouse cell lines.

Caki-1 cells (human kidney carcinoma) were treated with 0, 5, 10, 25, 50 μM $BeSO_4$ or 5, 15 mM LiCl for 24 hr. Caki-1 cells (from the DRC data) are more sensitive to $BeSO_4$ and LiCl, hence the maximum concentrations of beryllium and lithium was limited to 50 μM and 15 mM respectively. The cell lysates were analyzed for the Ser-9 phosphorylation of GSK-3β through western blotting (#9336S, rabbit polyclonal antibody). Increase in the Ser-9 phosphorylation of GSK-3β after treating the cells with increasing concentration of Be^{2+} was observed (Fig. 10b). Li^{+} also caused an increase in the Ser-9 phosphorylation of GSK-3β**.**

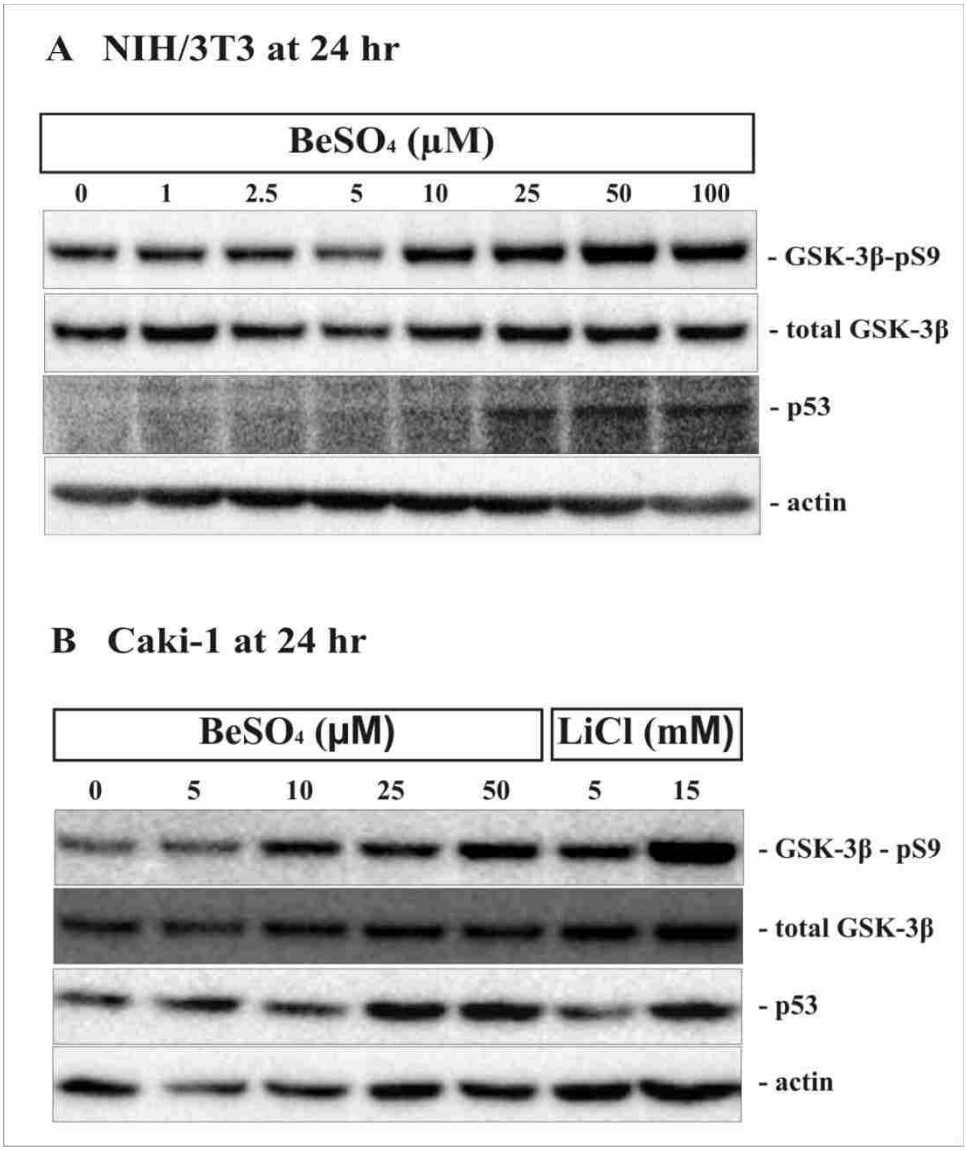


Fig. 10 Be^{2+} induces an increase in the Ser-9 phosphorylation of GSK-3β in NIH/3T3 and Caki-1 cells. a-b, western blots showing the Ser-9 phosphorylation of GSK-3β (**GSK-3β-pS9)** was detected by western blotting using phospho-GSK-3β (Ser-9) antibody. Total GSK-3β and actin protein levels are shown as loading control. p53 has been used to validate the effect of Be^{2+} on the cells. **a** NIH/3T3 cells treated with 0, 1, 2.5, 5, 10, 25, 50 or 100 μM $BeSO_4$ for 24 hr. **b** Caki-1 cells treated with 0, 5, 10, 25 or 50 μM $BeSO_4$ or 5, 15 mM LiCL for 24 hr.

To verify the results of pSer9-GSK-3β in NIH/3T3 cells, an alternate analytical method flow cytometry was used to quantify the Ser-9 phosphorylation of GSK-3β in situ (Fig. 11). Be^{2+} (a) and Li^{+} (b) treatment of NIH/3T3 cells at 24 hr produced a concentration dependent increase in the Ser-9 phosphorylation of GSK-3β corroborating the western results. These flow results suggests that in NIH/3T3 cells Be^{2+} is imitating Li^{+} by inducing an increase in the inhibitory post translational modification of GSK-3β i.e. Ser-9 phosphorylation of GSK-3β.

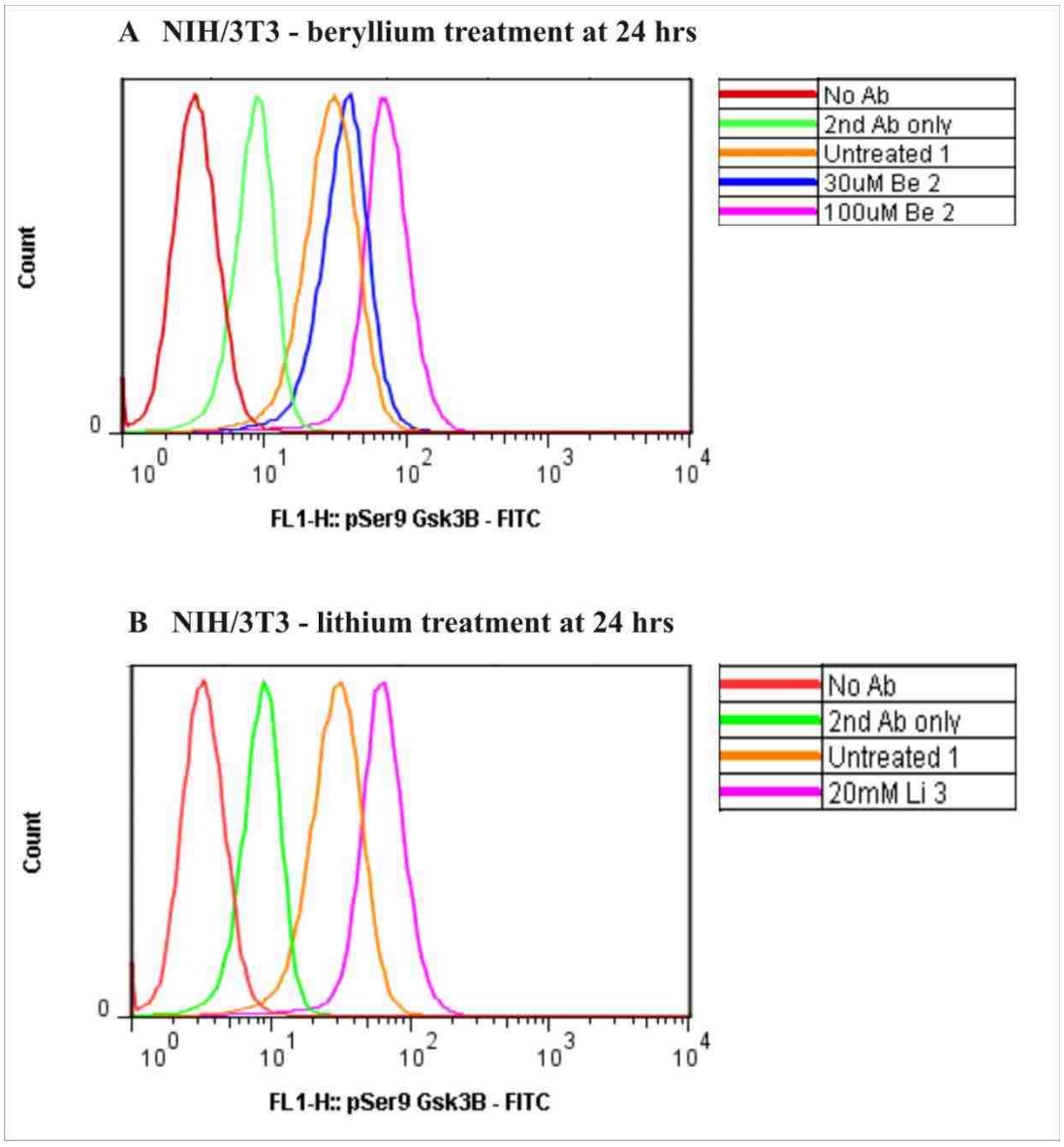


Fig. 11 Be^{2+} treatment induces an increase in the Ser-9 phosphorylation of GSK-3β in NIH/3T3 cells – a flow cytometric approach. The increase in the Ser-9 phosphorylation of GSK-3β in NIH/3T3 cells in the presence of beryllium and lithium was measured by flow cytometry. **a** flow cytometry histograms of NIH/3T3 cells treated with 0, 30 or 100 µM $BeSO_4$ for 24 hr. **b** flow cytometry histograms of NIH/3T3 cells treated with 0 or 20 mM LiCl for 24 hr. GSK-3β-pS9 (#5558 (D85E12), rabbit polyclonal) and FITC conjugated secondary antibody was used. For each beryllium and lithium treatment, mean fluorescence per cell was determined. Data shown here is representative of at least two independent experiments done in triplicates with similar results.

Different GSK-3β inhibitors induce an identical or matching effect on the phosphorylation status of GSK-3β, whereas beryllium's effect on Ser-9 phosphorylation of GSK-3β is cell type specific

Post translational modification of GSK-3β (Ser-9 phosphorylation of GSK-3β) plays an important role in its regulation. An increase in the Ser-9 phosphorylation of GSK-3β in beryllium treated NIH/3T3 and Caki-1 cells was observed in our study. On the other hand A172, HFL and U87MG cells seem to be immune to the beryllium induced Ser-9 phosphorylation of GSK-3β. It is important to investigate whether any other GSK-3β inhibitor can induce Be^{2+} like cell type specific effect on the Ser-9 phosphorylation of GSK-3β. The effect of Be^{2+} was compared against lithium, SB216763, rottlerin, $CaCl_2$ and insulin in A172, NIH3T3, and Caki-1 cells.

Insulin is a negative regulator of GSK-3β and it induces an increase in its inhibitory Ser-9 phosphorylation. It is possible that only certain cell lines would be able to respond to insulin treatment. The insulin receptor is widely distributed in mammalian tissues, and it was anticipated that each of these cell types might be suitable for such a comparison. A172 cells are derived from human glioma, and human glioma cells are known to express insulin receptors (Grunbereger et al., 1986) Normal untransfected serum-starved NIH3T3 cells have been shown to respond to insulin (Bossenmaier et al., 2000). The Caki-1 are renal carcinoma cells and kidney is a major organ for glycogen storage that is highly responsive to insulin. It can be expected that these three cell lines should be able to respond to insulin.

Rottlerin is known to cause an increase in the Ser-9 phosphorylation of GSK-3β (Gschwendt et al., 1994; Zhang et al., 2003). *Zhang et al* have used rottlerin at 5 μM to analyze its effect on the Ser-9 phosphorylation of GSK-3β in Neuro2A cells. We decided to use rottlerin at 10 μM for A172 cells (to maximize the effect) and considering the fact that Caki-1 cells are sensitive to GSK-3β inhibitors the rottlerin concentration was fixed at 5 μM for this study. Another small

molecule inhibitor of GSK-3β is SB216763 - an anilino malemide (Coghlan et al., 2000) was also selected for this study. Dose response curve of A172, NIH/3T3 and Caki-1 cells with SB21673 was used to decide the SB216763 dosage to be used for the given cell lines 10 µM for Caki-1 and 20 µM for both A172 and NIH/3T3 (Appendix-VI). In addition the effect of insulin, LiCl and $CaCl_2$ on the Ser-9 phosphorylation of GSK-3β was compared parallely with $BeSO_4$ in NIH/3T3, Caki-1 and A172 cells. In our studies insulin was found to be effective within a range of 100-200 nM.

As observed previously $BeSO_4$ at 24 hr did not induce any increase in the Ser-9 phosphorylation of GSK-3β in A172 cells (Fig. 12, panel c) conversely an increase in the Ser-9 phosphorylation of GSK-3β in NIH/3T3 (panel a) and Caki-1 cells (panel b) is observed. Lithium – LiCl at 24 hr induced an increase in the Ser-9 phosphorylation of GSK-3β in all the three cell lines, A172 (panel c), NIH/3T3 (panel a) and Caki-1 (panel b). To analyze the effect of insulin on phosphorylation status of GSK-3β, cells were serum starved as mentioned in the materials and methods section. After inducing cells with 200 nM of Insulin for 30-60 min, an increase in the Ser-9 phosphorylation of GSK-3β is observed in NIH/3T3 (panel a) and a slight increase in A172 (panel c) is observed. The effect of insulin on Caki-1 cells is not clear (panel b). Like lithium, insulin too produces an identical effect i. e. increase in Ser-9 phosphorylation of GSK-3β in NIH/3T3 and A172 cell lines. Rottlerin at 10 µM and 5 µM for 24 hr produced inconsistent results in A172 (panel c) and Caki-1 (panel b) cells respectively. Rottlerin at 10 µM seems to be causing a slight increase in Ser-9 phosphorylation in NIH/3T3 cells (panel a). Overall the effect of rottlerin on the Ser-9 phosphorylation is inconclusive. SB216763 at a concentration of 10-20 µM caused a drastic decrease in Ser-9 phosphorylation of GSK-3β in NIH/3T3 (panel a), Caki-1 (panel b) and A172 (panel c). SB216763 induced decrease in the Ser-9 phosphorylation of GSK-3β is an unexpected result, which is quite different from the "inhibitory effects" of other GSK-3β inhibitors. In all the three cell lines the total Gsk-3β levels are stable indicating that SB216763 is

inducing a decrease only in the phosphorylation status of GSK-3β. A possible explanation for SB216763 induced decrease in pSer9-GSK-3β could be because of its failure to deactivate phosphatases that in turn could be causing a drastic decrease in the pSer9-GSK-3β. Li^+ affects an increase in the Ser-9 phosphorylation of GSK-3β by modulating the activity of protein phosphatase 1 (PP1) (Zhang et al., 2003). SB216763 is a specific GSK-3β inhibitor with no known inhibitory activity against other related kinases (Coghlan et al., 2000). It can be speculated that SB216763 because of its specific activity towards GSK-3β only may not be able to inhibit the activity of PP1. The active PP1 could be inducing a drastic decrease in the Ser-9 phosphorylation of GSK-3β as observed in the SB216763 samples. However SB216763 produced identical results in three different cell lines (in relation to pSer9-GSK-3β); adding confidence in its ability to function as a reliable control. $CaCl_2$ at 100 µM did not induce any noticeable change in the Ser-9 phosphorylation of GSK-3β. It indicates that the up regulation of pSer9-GSK-3β is a property limited to specific GSK-3β inhibitors.

A comparison of the effect of different GSK-3β inhibitors on the Ser-9 phosphorylation of GSK-3β clearly shows that among the 5 different GSK-3β inhibitors used in this study i.e. $BeSO_4$, LiCl, Insulin and SB216763; only Be^{2+} elicits a clear cell type specific effect on the Ser-9 phosphorylation of GSK-3β (Table 5). Other GSK-3β inhibitors listed below elucidates their inhibitory effects either by inducing a definitive/identical increase (LiCl and insulin) or decrease (SB216763) in the Ser-9 phosphorylation of GSK-3β in the mentioned cell lines. These results suggest that Be^{2+} could be modulating its effect on the Ser-9 phosphorylation of GSK-3β via a unique and cell type specific mechanism.

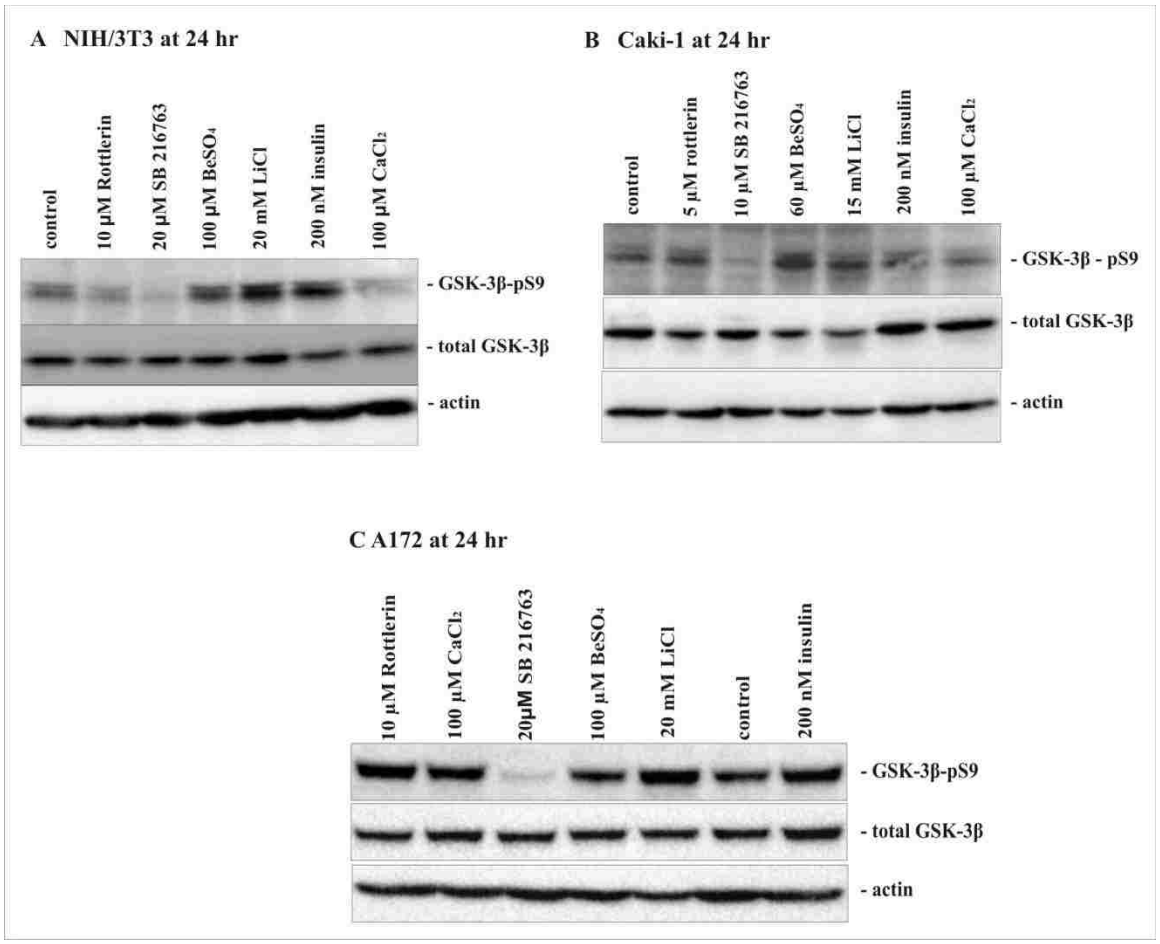


Fig. 12 Unique effect of beryllium on the Ser-9 phosphorylation status of GSK-3β compared to other established GSK-3β inhibitors and $CaCl_2$ salt. **a-c**, western blots showing the Ser-9 phosphorylation of GSK-3β (**GSK-3β-pS9)** was detected by western blotting using phospho-GSK-3β (Ser-9) antibody. Total GSK-3β and actin protein levels are shown as loading control. **a, c** NIH/3T3 and A172 cells were treated with 0, 100 µM $BeSO_4$, 20 µM SB216763, 20 mM LiCl, 100 µM $CaCl_2$ or 10 µM rottlerin (only for A172 cells) for 24 hr or 200 nM Insulin for 30-60 min. **b**, Caki-1 cells were treated with 0, 60 µM $BeSO_4$, 10 µM SB216763, 15 mM LiCl, 100 µM $CaCl_2$ or 5 µM rottlerin for 24 hr or 200 nM Insulin for 30-60 min.

Table 7. Effect of GSK-3β inhibitors and $CaCl_2$ salt on the Ser-9 phosphorylation status of GSK-3β

	$BeSO_4$	LiCl	Insulin	SB216763	Rottlerin	$CaCl_2$
A172	**No change**	**Increase**	**Increase**	**Decrease**	**Inconclu-sive***	**No change**
NIH/3T3	**Increase**	**Increase**	**Increase**	**Decrease**	**Inconclu-sive**	**No change**
Caki-1	**Increase**	**Increase**	**Increase**	**Decrease**	**Inconclu-sive***	**No change**

* Effect of rottlerin on the pSer9-GSK-3β was inconsistent; hence it was difficult to ascertain the role played by it in relation to the inhibitory Ser-9 phosphorylation of GSK-3β.

Induction of Ser-9 phosphorylation on GSK-3β by $BeSO_4$ is not random salt effect

To establish that the Be^{2+} induced increase in Ser-9 phosphorylation of GSK-3β in NIH/3T3 cells is not a random salt effect, NIH/3T3 cells were treated with 0 or 100 μM $BeSO_4$, KCl, $MgCl_2$ or Na_2SO_4 for 24 hr (Fig. 13). The lack of increase in the Ser-9 phosphorylation of GSK-3β in the 100 μM KCl, $MgCl_2$ or Na_2SO_4 treated samples confirms the Be^{2+} induced increase in Ser-9 phosphorylation is not a random salt effect.

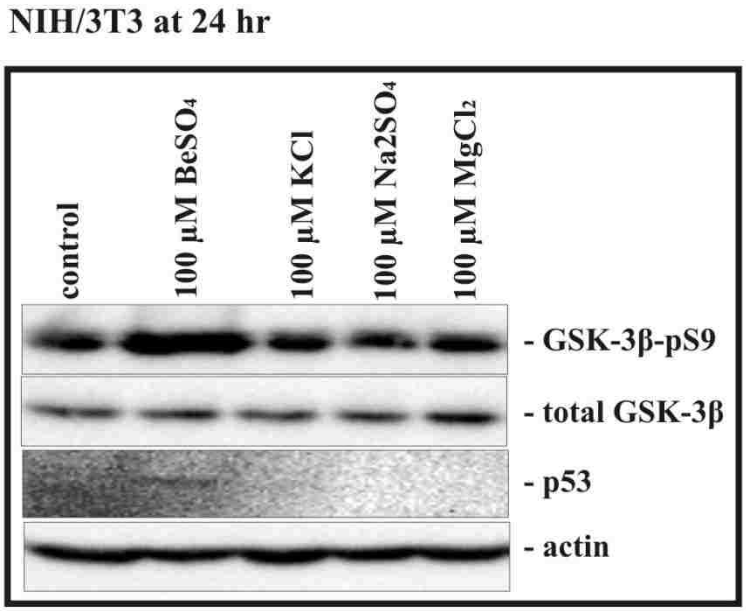


Fig. 13 B**eryllium induced Ser-9 phosphorylation in NIH/3T3 is not random salt effect.** . NIH/3T3 cells were treated with 0 or 100 µM $BeSO_4$, KCl, $MgCl_2$ or Na_2SO_4 for 24 hr.Western blots showing the Ser-9 phosphorylation of GSK-3β (**GSK-3β-pS9)** was detected by using phospho-GSK-3β (Ser-9) antibody. Total GSK-3β and actin protein levels are shown as loading control. p53 has been used to validate the effect of Be^{2+} on the NIH/3T3 cells

Investigating the credentials of beryllium as a biologically active GSK-3β inhibitor

Be^{2+} induced down regulation in the phosphorylated form of glycogen synthase enzyme validates its intra cellular effect

Glycogen synthase (GS) enzyme plays a central role in glycogen metabolism. There are several kinases that regulate the activity of GS by modulating its phosphorylation. The moniker "glycogen synthase kinase – 3" was coined because of the ability of GSK-3 to phosphorylate glycogen synthase enzyme. GSK-3β phosphorylates GS and plays an important role in repressing its activity (Lawerence and Roach, 1997). There is enough evidence to demonstrate the important role played by GSK-3β in glycogen metabolism because of its ability to regulate GS. The phosphorylation sites which play an important role in the activation or de-activation of the GS are target sites of GSK-3β (Rylatt et al., 1980; Parker et al., 1983).

Since the activity of GS is regulated by GSK-3β any event or inhibitor, which can regulate the activity of GSK-3β is expected to have an affect on GS as well. The positive effect of Li^{+} - an established GSK-3β inhibitor on the stimulation of glycogen synthase and glycogen synthesis has been proved in various studies (Cheng et al., 1983; Tabata et al., 1994; Orena et al., 2000; Furnsinn et al., 1997; Chen et al., 1998). *MacAulay et al* demonstrated that exposure of L6 muscle cells and 3T3-L1 adipocytes to Li^{+} leads to accumulation of de-phosphorylated GS (active form of GS) (MacAulay et al., 2003).

Be^{2+} is a potent GSK-3β inhibitor, but the inhibitory effect of Be^{2+} on the ability of GSK-3β to phosphorylate its bonafide substrates has not been demonstrated yet. Be^{2+} seems to be inducing an increase in the Ser-9 phosphorylation of GSK-3β in NIH/3T3 and Caki-1 cells (Fig. 12). The phosphorylated Ser-9 residue on the N-terminal region acts as pseudo-substrate of GSK-3β thereby blocking its active site (Dajani et al., 2001). It can be hypothesized that the inactivated GSK-3β (blocked active site) will not be able to phosporylate its substrate i.e. the GS enzyme

hence a decrease in the pGS levels may be observed. In this study the effect of Be^{2+} on glycogen synthase (GS) an important GSK-3β substrate was investigated in cell culture.

Selecting the best cell line for glycogen synthase studies

In order to select a cell line with optimum expression of glycogen synthase (GS); A172, Caki-1 and NIH/3T3 were cultured in the presence of 0 or 20 mM LiCl for 24 hr. Total cell lysates of these cells lines were probed for the expression of pGS. Caki-1 and A172 samples seems to be having no visible pGS bands indicating that these two cell lines may not be suitable for GS studies (Fig. 14). NIH/3T3 cells show optimum expression of GS and also seem to be responsive to inhibitory effects of LiCl. Hence NIH/3T3 cells were selected for further pGS studies.

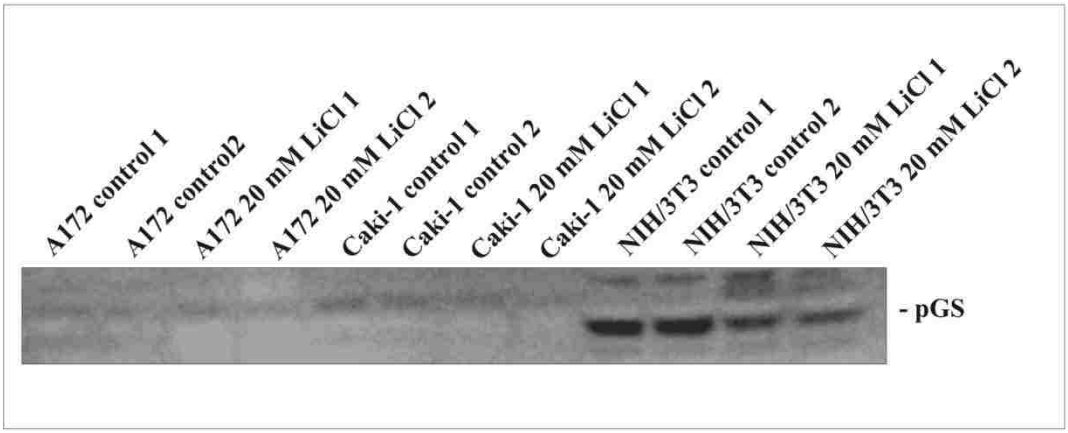


Fig 14: **Murine fibroblasts are responsive to LiCl induced GS dephosphorylation**.Total cell lysates extracted from A172 , Caki-1and NIH/3T3 treated with 0 or 20 mM LiCl for 24 hr were analysed using western blotting for the pGS levels. The pGS (Ser641/Ser645) antibody #07-817 from millipore was used.

Effect of Be^{2+} on pGS in murine fibroblasts (NIH/3T3 cells)

NIH/3T3 cells were treated with 0, 30 or 100 µM $BeSO_4$ or 20 mM KCl or 20 mM LICl or 20 µM SB216763 for 24 hr or serum starved NIH/3T3 cells were induced with 100 nM insulin for 15–60 min. The phosphorylation state of glycogen synthase (GS) in the total cell lysates was analyzed by western blotting using a phospho specific affinity purified rabbit antibody from Millipore (#07-817). The GSK-3β inhibitors Be^{2+}, Li^{+} and SB216763 caused a decrease in the phospho-glycogen synthase levels. As expected no decrease in the pGS levels was observed in the KCl treated samples (KCl was intended to serve as a negative control) (Fig. 15a).

This data suggests that Be^{2+} treatment elicits a decrease in the pGS levels of NIH/3T3 cells, demonstrating the ability of beryllium to inhibit the intra cellular kinase activity of GSK-3β in cell culture.

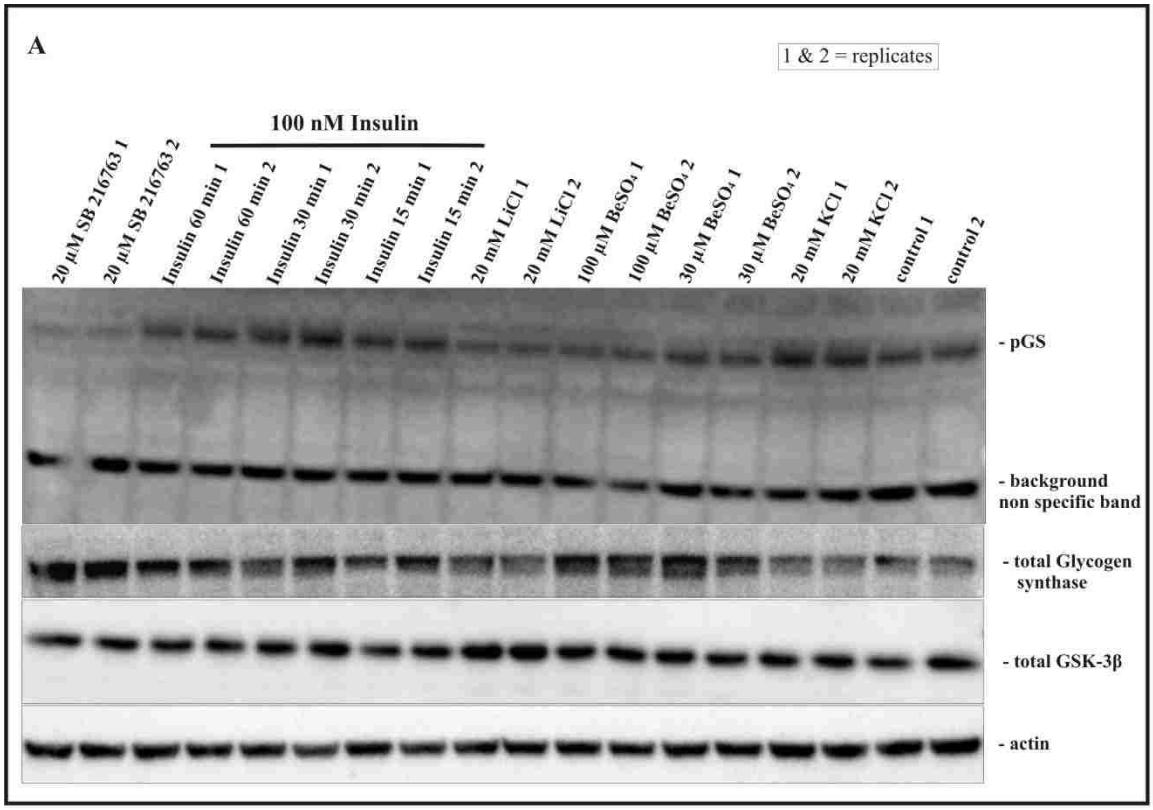


Fig. 15a GSK-3β inhibitors and beryllium induce a decrease in the phospho-GS. NIH/3T3 cells were cultured in the presence of 0 or 30 or 100 µM $BeSO_4$ or 20 mM LiCl or 20 µM SB216763 or 20 mM KCL. For insulin treatment cells were grown in RPMI media supplemented with 10% bovine growth serum for 24 hr. Post 2X washing with serum less RPMI cells were serum starved by growing them in 0% BGS RPMI for 2 - 2.5 hrs. Cells were induced with 100 nM insulin for 15 or 30 or 60 min. Total cell lysates were analyzed for phospho-GS using a affinity purified rabbit polyclonal antibody. The non specific low molecular weight back ground band in the pGS panel serves as an internal loading control. Total GS, actin and total GSK-3β were used as loading controls.

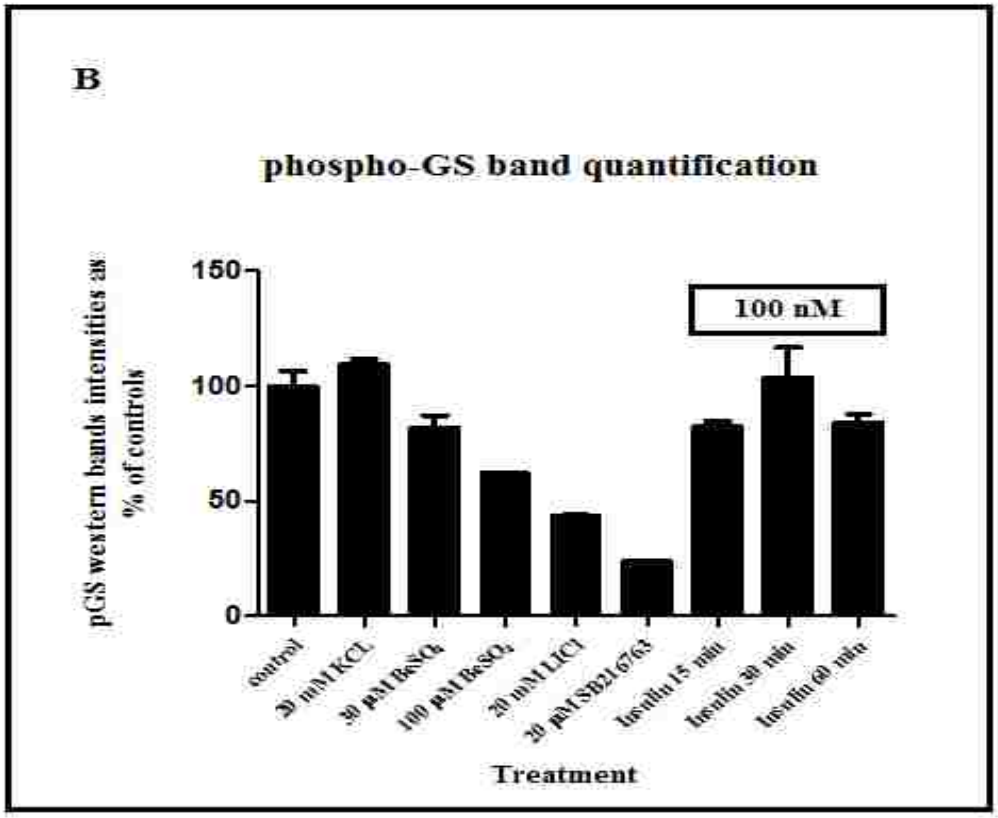


Fig 15b phospho-GS band intensity quantification. The phospho-GS bands intensity was quantified using Licor ImageStudio 5.0. The band intensity was plotted as percentage of control. (Additional information about band quantification is available in Appendix VII)

Closely spaced pGS bands in murine fibroblast samples

An interesting observation in the phospho-GS westerns has been the appearance of a cluster of three closely spaced bands as seen on the pGS blots at molecular weight close to that of glycogen synthase i.e. ~80-90 kDa. These bands have been generically designated as band 1, band 2 and band 3 (Fig. 16a). GSK-3 phosphorylates glycogen synthase (GS) on different serine residues designated as Ser-640, 644, 648 and 652 (Rylatt et al., 1980; Parker et al., 1983). GS is primed at Ser-656 by casein kinase II (CKII) (Picton et al., 1982) and is then sequentially phosphorylated at Ser-652 followed by Ser-648 and so on (Fiol et al., 2000). According to *Fiol et al* the phosphorylation of GS by GSK-3β is not random, phoshorylation of Ser-652 leads to phoshorylation of Ser-648, phosphorylation of Ser-648 leads to phosphorylation of Ser-644 and so on.

The consensus sequence for the phosphorylation sites of GSK-3β in rabbit skeletal muscle cells is -P-R-P-A-**S**(Ser-640/641)-V-P-P-**S**(Ser-644/645)-P-S-L-S(Ser-648)-R-H-S-**S**(Ser-652)-P-H-Q-S(Ser-656)-E-D-E-E-P (Ryllat et al., 1980). The amino acids of GS mentioned in bold are the target sites of GSK-3β. Majority of the initial work related to GS was carried out in rabbit muscle cells and the amino acid numbers were designated based on biochemical studies. The work on mouse and human GS is more recent and follows the HUGO recommendations; that begin with the translation start site. Hence the amino acid numbers between rabbit GS and mouse/human GS differ by one i.e the Ser-640 of rabbit GS (in the consensus sequence) corresponds to the Ser-641 of mouse GS (explained in Roach et al., 2012).

For western blots, an affinity-purified rabbit polyclonal antibody specific for mouse or human GS was used. The immunogen used for raising the phospho-GS-antibody is a synthetic peptide containing phosphor-serines at residues 641 and 645 in the human GS sequence. A cluster of closely spaced bands is apparent in the phospho-GS western blot (Fig. 16a). Among the three

bands, band 2's intensity is decreasing in response to GSK-3β inhibitors as indicated by the line label. Band 2 was interpreted as representing the main product of GSK3 phosphorylation, because it was sensitive to treatment with GSK3 inhibitors. It is unclear whether the flanking bands represent background bands, or alternative forms of phospho-GS arising from the availability of multiple phosphorylation sites. Fortuitously, phospho-GS western blots also contain a background band that runs in a different region of the gel, at much lower MW – indicated by an arrow. This band was included in the western images to demonstrate the sharp resolution attained during electrophoresis, and also so that it could serve as an extra loading control to document equivalent total protein per lane.

Alternatively it is also possible that the closely spaced bands are representing the different isoforms of glycogen synthase protein. In mammals two isoforms of glycogen synthase are expressed: glycogen synthase -1 (GYS1) and glycogen synthase-2 (GYS2). GYS1 is expressed mostly in skeletal muscle and most other cell types and GYS2 is restricted to liver (Browner et al., 1989; Nuttall et al., 1994). It has been observed that NIH/3T3 cells could possibly be expressing both the isoforms of glycogen synthase because of its embryonic lineage. There is no direct evidence to suggest that NIH/3T3 cells indeed express both the isoforms of glycogen synthase. However phospho-glycogen synthase antibodies from different sources seem to be identifying both the isoforms of glycogen synthase in NIH/3T3 total cell lysates (Appendix IX). Depending upon the information available from the use of antibodies from different sources in NIH/3T3 cells it can be speculated that indeed NIH/3T3 cells could possibly be expressing both the isoforms of glycogen synthase. It is observed that lithium, beryllium and SB216763 seem to induce a decrease in the phosphorylation status of band 2. From the molecular weight difference between the two isoforms of glycogen synthase it can be speculated that band 2 is representing glycogen synthase-1 (Fig. 16a). The effect of insulin on the pGS is not clear, possibly because of the failure of serum starvation or partly because NIH/3T3 cells may not be the perfect system to

analyze the insulin-pGS dynamics. However the GSK-3β inhibitors included in this study seem to have little effect on the phosphorylation status of glycogen synthase-2 (band 3).

The GSK-3β inhibitors Be^{2+}, Li^{+}, insulin (* effect on band 2 not clear) and SB216763 caused a decrease in the pGS levels (Fig 16b). Correspondingly an increase in the pSer-9 of GSK-3β induced by all the GSK-3β inhibitors except for SB216763 was observed. SB216763 induced a decrease in the pGS but did not induce an increase in the pSer9-GSK-3β. KCL at 20 mM concentration did not induce any major change in the pSer9-GSK-3β signal or decrease in the pGS levels. This data supports the observation that Be^{2+} treatment elicits a decrease in the pGS levels of NIH/3T3 cells.

These results demonstrate the ability of beryllium to inhibit intra cellular kinase activity of GSK-3β in cell culture. This is first time ever the effect of beryllium on a GSK-3β substrate has been documented in cell culture.

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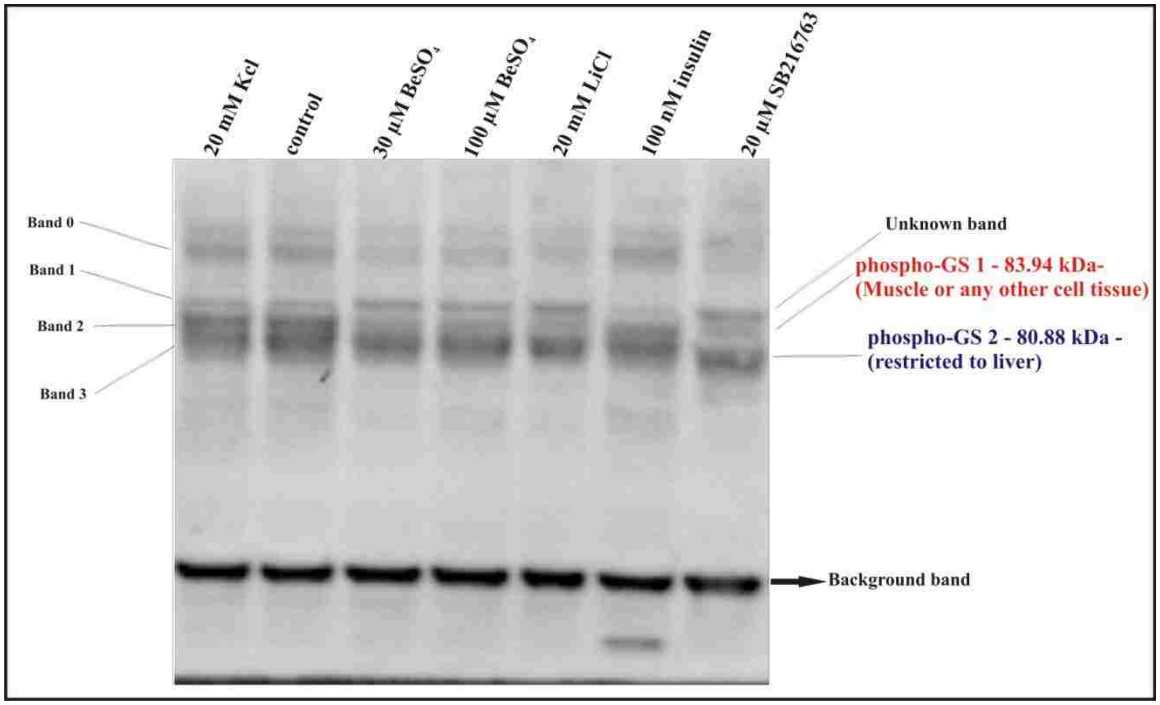


Fig. 16a Identification of the correct pGS band in the multiple pGS bands cluster. Cell lysates extracted from NIH/3T3 cells treated with 0, 30 or 100 µM $BeSO_4$ or 20 mM KCL or 20 mM LICl or 20 µM SB216763 for 24 hr or serum starved NIH/3T3 cells induced with 100 nM insulin for 30 – 60 min were assessed for the effect of different GSK-3β inhibitors on the pGS levels. Western blots showing the protein levels of pGS.

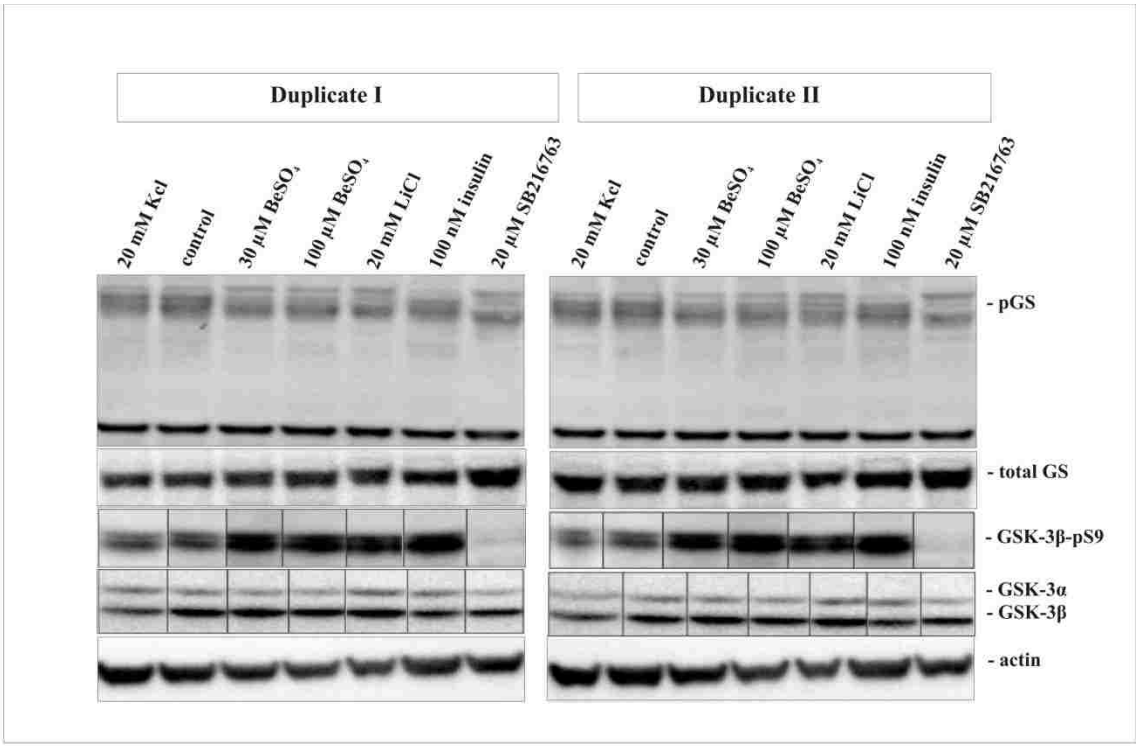


Fig 16b Effect of different GSK-3β inhibitors on phospho-GS. Cell lysates extracted from NIH/3T3 cells treated with 0, 30 or 100 µM $BeSO_4$, 20 mM KCL, 20 mM LICl, 20 µM SB216763 for 24 hr or serum starved NIH/3T3 cells induced with 100 nM insulin for 30 – 60 min were assessed for the effect of different GSK-3β inhibitors on the pGS levels. Western blots showing the protein levels of pGS. Note: The total GSK-3α/β and pSer9—GSK-3β bands were re-arranged to match the lane sequence of the accompanying blots.

Beryllium has little effect on the Wnt signaling pathway compared to other GSK-3β inhibitors

β-catenin is an important substrate of GSK-3β wherein GSK-3β-mediated phosphorylation of β-catenin leads to its ubiquitin-mediated proteosomal degradation (Miller and Moon, 1996; Aberle et al., 1997; Ikeda et al., 1998). GSK-3β and β-catenin play a central role in the Wnt signaling pathway; inactivation of GSK-3β is known to stabilize β-catenin and lead to its accumulation in the nucleus (Polakis, 2000; Lustig and Behrens, 2003). Li^+ treatment induces an increase in the N-terminal dephosphorylation of β-catenin which is then translocated to the nucleus (van Noort et al., 2002; Staal et al., 2002; Hagen et al., 2002). In order to understand the effect of Be^{2+} on the Wnt signaling pathway, we investigated whether Be^{2+} treatment can cause an increase in the nuclear pool of β-Catenin.

Compared to Li^+, Be^{2+}- treatment induces negligible nuclear localization of β-Catenin in NIH/3T3 cells

We optimized confocal microscopy to study the effect of $BeSO_4$ on the nuclear localization of β-catenin. The effect of $BeSO_4$ along with other GSK-3β inhibitors like insulin, LiCl or SB216763 was analyzed in NIH/3T3 cells (Fig**.** 17a). The control NIH/3T3 cells show that the β-catenin is predominantly present in the cytoplasm (Fig 17a, lane 1). 20 mM LiCl led to nuclear accumulation of β-catenin in NIH/3T3cells as indicated by the arrows (Fig 17a, lane 2). Li^+ mimics the effect of Wnt ligands (Stambolic et al., 1996; Hedgepeth et al., 1997) and leads to accumulation of β-catenin in the nucleus. 20 μM SB216763 also caused nuclear localization of β-catenin (Fig 17A, lane 4). For stimulation with insulin, NIH/3T3 cells were serum starved and stimulated with 200 nM insulin for 30 – 60 min. The nuclear localization pattern of β-catenin in NIH/3T3 cells treated with 200 nM insulin and 100 μM $BeSO_4$ is comparable to the control cells. The lack of much nuclear localization of β-catenin is a hallmark of the control cells. Insulin is a

GSK-3β inhibitor which doesn't affect the Wnt signaling pathway (Ding et al., 2000). The morphology and nuclear β-catenin localization pattern of NIH/3T3 cells treated with $BeSO_4$ and insulin are similar to the control cells indicating that Be^{2+} could be mimicking insulin's mode of inhibitory action on GSK-3β.

(Note: Refer supplementary figures I on page-186 for individual images)

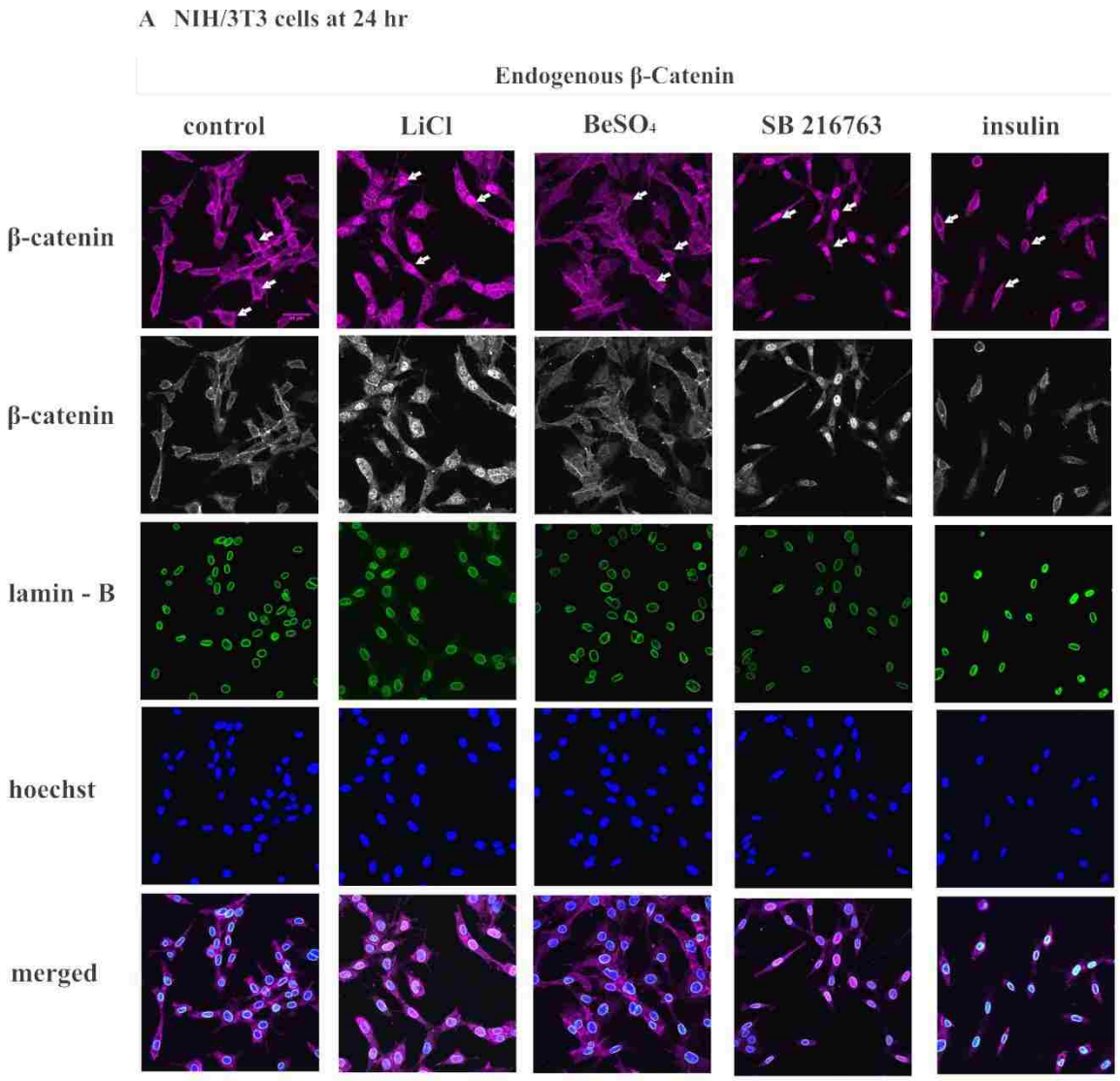


Fig. 17a Effect of Be^{2+} on the nuclear localization of β-catenin in NIH/3T3 cells. Immunofluorescence microscopy images of NIH/3T3 cells treated with 0, 100 µM $BeSO_4$, 20 mM LiCl, 20 µM SB216763 for 24 hr or stimulated with 200 nM insulin for 30 – 60 min after serum starvation. The cells were fixed with 4% formalin and double labeled with a mouse monoclonal antibody specific for β-catenin and a goat polyclonal anytibody specific for lamin-B followed by anti mouse Alexa-647/anti goat-FITC secondary antibodies. Nuclear localization of β-catenin in the LiCl and SB216763 treated NIH/3T3 cells is seen (lane 2, 4 respectively) whereas not much accumulation of nuclear β-catenin is observed in the insulin and $BeSO_4$ treated cells (lane 3, 5). All the images are shown at x40 magnification. Scale bar set at 50 µm.

Be^{2+} induces minimal nuclear localization of β-catenin in A172 and A172 cells over expressing wt GSK-3β

A172 cells were treated with 0, 100 μM $BeSO_4$ or 20 mM LiCl or 20 μM SB216763 for 24 hr. Control A172 cells show a conspicuous hollow nucleus (indicated by arrows) indicating the cytoplasmic localization of β-catenin (Fig. 17b)**.** Li^+ and SB216763 induced a clear localization of β-catenin in the nucleus (lane 2, 4). Be^{2+} did not elicit any drastic change in the nuclear localization of β-catenin and the Be^{2+} treated cells are similar to the control cells in terms of nuclear localization of β-catenin (lane 3). These results indicate that Be^{2+} has minimal effect on nuclear localization of β-catenin in A172 cells when compared to other GSK-3β inhibitors.

A172 over expressing wt GSK-3β can act as a valuable system to analyze the effects of GSK-3β-inhibition. The abundant amount of GSK-3β could be helpful in demonstrating the after effects of GSK-3β-inhibition compared to untransfected A172 cells expressing native/normal levels of GSK-3β. A172 cells were transfected with the addgene clone #14753 to over express wt GSK-3β. Single cell clones of transfected A172 cells over expressing wt GSK-3β were selected. A172 clone over expressing wt GSK-3β named as P1 (Appendix V) was selected for the immuno fluorescence imaging. P1B cells (A172 cells over expressing wt GSK-3β) were treated with 0, 100 μM $BeSO_4$ or 20 mM LiCl for 24 hr. Control cells show conspicuous empty nucleus (arrows) indicating the cytoplasmic localization of β-catenin (Fig**.** 17c**,** lane 1)**.** Li^+ treatment induced a clear localization of β-catenin in the nucleus (lane 2). Be^{2+} did not elicit any drastic change in the nuclear localization of β-catenin and the Be^{2+} treated cells are somewhat similar to the control cells in terms of nuclear localization of β-catenin (lane 3). These results coupled with the data from untransfected A172 (Fig. 17b) cells indicate that Be^{2+} has minimal inhibitory effects on Wnt signaling pathway in A172 cells compared to other established GSK-3β inhibitors. The confocal data from NIH/3T3, A172 and A172 cells over expressing wt GSK-3β (P1B) shows that probably Be^{2+} has nil to minimal effect on the Wnt signaling pathway.

(Note: Refer supplementary figures II on page-191 for individual images)

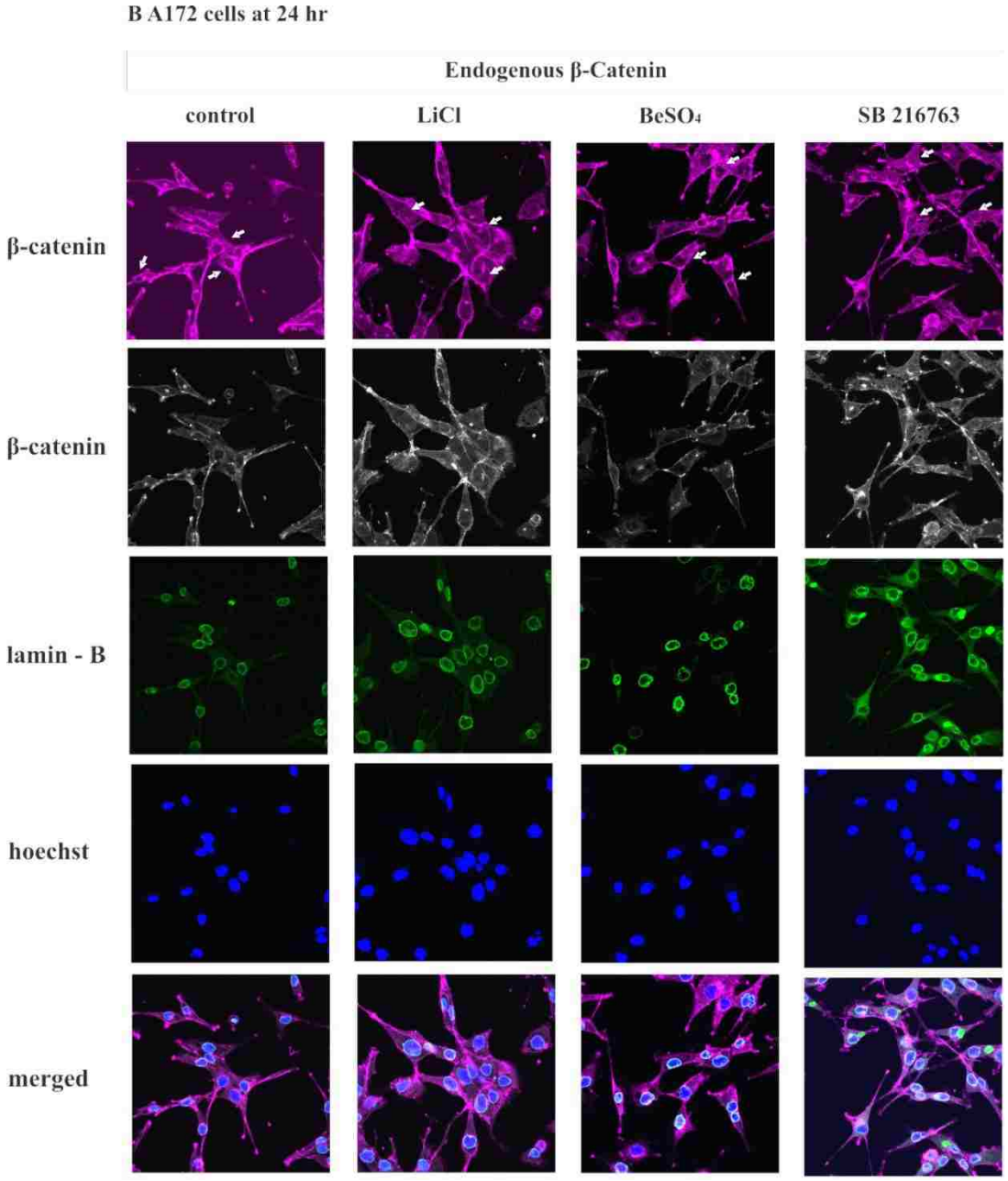


Fig. 17b The effect of Be^{2+} on nuclear localization of β-catenin in A172 cells. Immunoflorescence microscopy images of A172 cells treated with 0, 100 µM $BeSO_4$, 20 mM LiCl or 20 µM SB216763 for 24 hr. The cells were fixed with 4% formalin and double labeled with a mouse monoclonal antibody specific for β-catenin and a goat polyclonal anytibody specific for lamin-B followed by anti mouse Alexa-647/anti goat-FITC secondary antibodies. Scale bar set at 50 µm.

(Note: Refer summplementary figures III on page–195 for individual images)

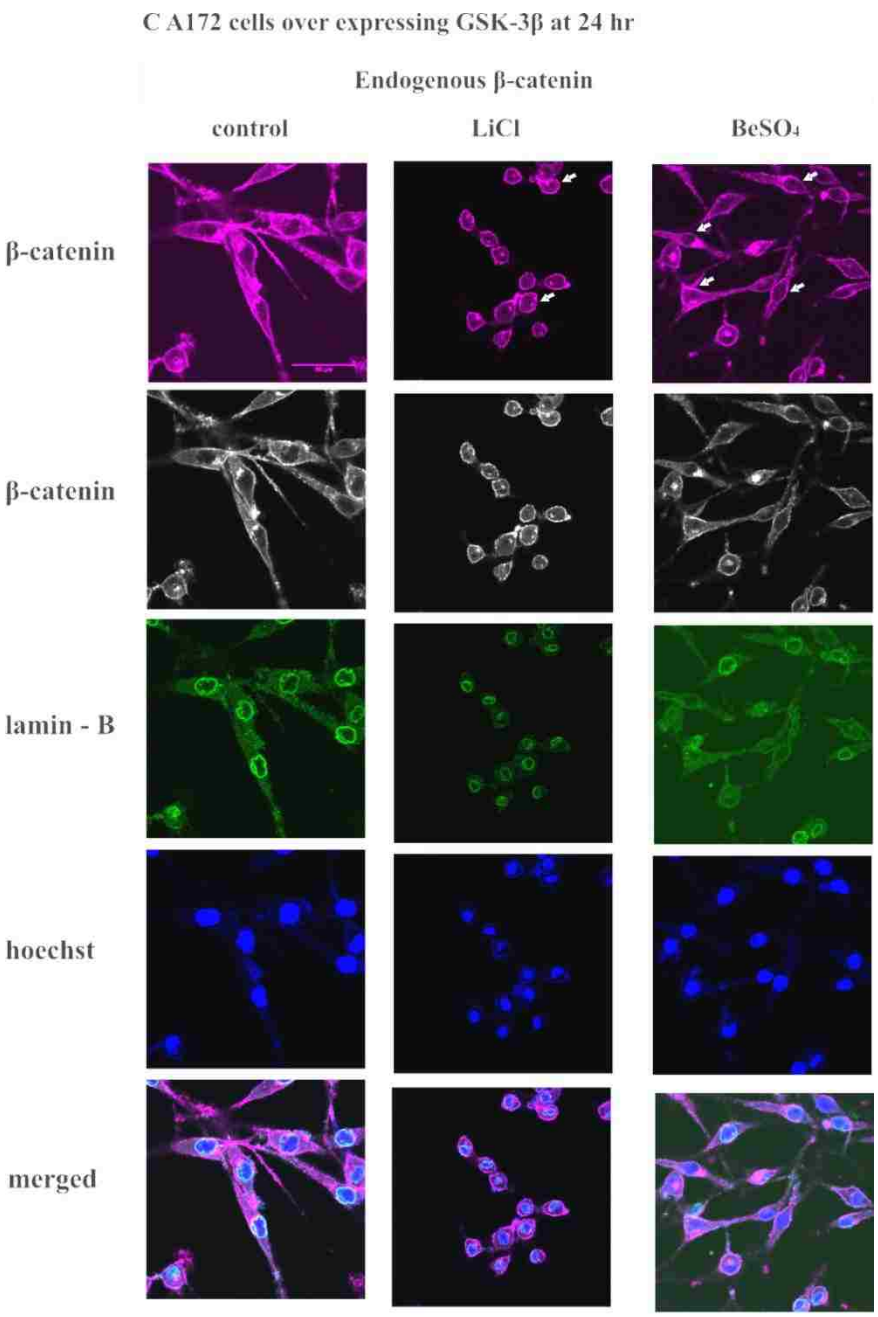


Fig. 17c Effect of Be^{2+} on the nuclear localization of β-catenin in A172 cells over expressing wt GSK-3β. Immunoflorescence microscopy images of A172 cells wt GSK-3β treated with 0, 100 µM $BeSO_4$ or 20 mM LiCl for 24 hr. The cells were fixed with 4% formalin and double labeled with a mouse monoclonal antibody specific for β-catenin and a goat polyclonal antibody specific for lamin-B followed by anti mouse Alexa-647/anti goat-FITC secondary antibodies. Scale bar set at 50 µm.

Nuclear localization of β-catenin in stable nuclei isolated from A172 cells – A flow cytometric approach

The immuno fluorescence imaging experiments showed that Be^{2+} had minimal effect on the nuclear localization of β-catenin whereas other types of GSK-3β inhibitors such as Li^{+} and SB216763 were effective. Assessing nuclear localization of β-catenin in the stable nuclei isolated from A172 cells was used as an alternative approach to analyze the effect of Be^{2+} on Wnt signaling pathway. A172 cells were treated with 100 μM $BeSO_4$ or 20 mM LiCl and nuclei were isolated from the cells (Fig 18). The isolated nuclei are stable for more than 24 hr at 4℃ as (explained in the materials and methods section).

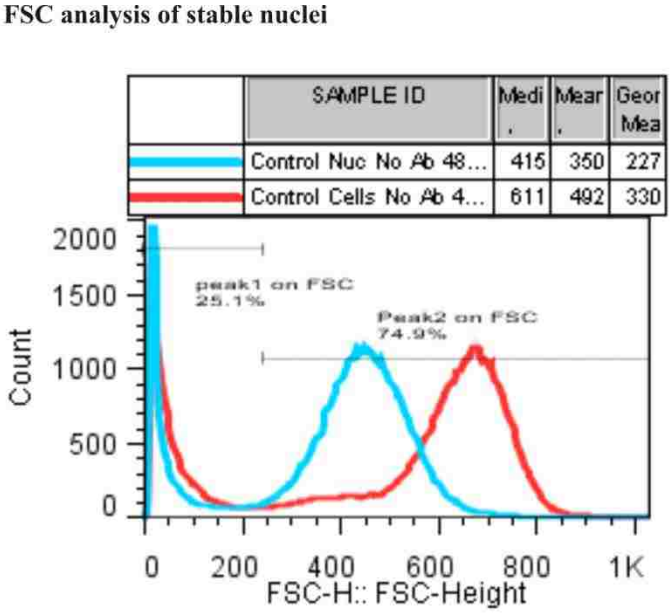


Fig. 18 FSC analysis of stable nuclei and formalin fixed A172 cells. A clear size difference between nuclei and cells is observed. The position of the peak (red) representing A172 cells on the x-axis demonstrates the fact that the isolated nuclei are smaller than the cells.

Be^{2+} treatment did not have a profound effect like Li^{+} on the nuclear localization of β-catenin

A172 cells treated with $BeSO_4$ or LiCl for 24 hr were used for isolating stable nuclei. Concentration dependent increase in the localization of β-catenin was observed in the nuclei isolated from Li^{+} treated A172 cells (Fig. 19b). A slight increase in the nuclear β-catenin is observed between the control nuclei and the Be^{2+} treated nuclei (Fig 19a). The effect of Be^{2+} on the nuclear localization of β-catenin in A172 cells is quite subdued in comparison to the results produced by Li^{+} treatment (Fig 19b).

The flow cytometry results along with the confocal imaging results indicate that Be^{2+} has minimal effect on the Wnt signaling pathway in NIH/3T3 and A172 cells, compared to other GSK-3β inhibitors like Li^{+} or SB216763.

a Analysis of stable nuclei isolated from A172 cells treated with $BeSO_4$

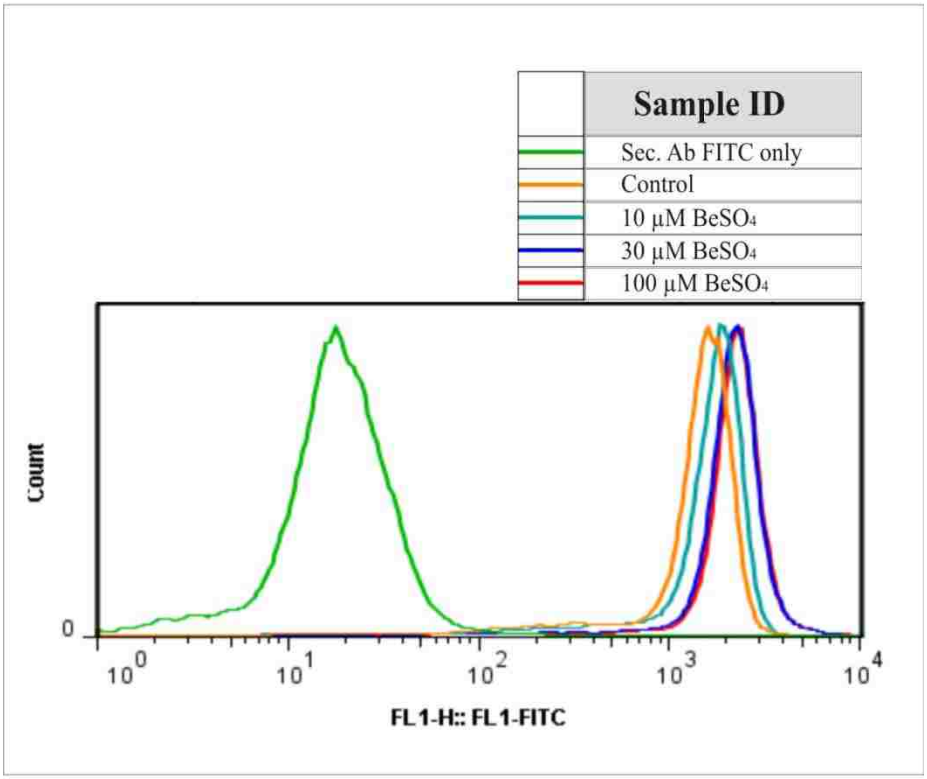


Fig. 19a The effect of Be^{2+} on nuclear localization of β-catenin is minimal compared to Li^{+}. A172 cells were treated with 0, 10, 30 or 100 µM $BeSO_4$ for 24 hr. Stable nuclei were extracted from the Be^{2+} treated cells. The effect of Be^{2+} on the nuclear localization of β-catenin was assessed using a mouse mono clonal β-catenin primary antibody and an Alexa647 – conjugated secondary antibody. For each treatment, mean fluorescence per cell was determined from independent replicates and representative peak for each treatment is shown here. The x-axis values are represented in log scale.

b Analysis of stable nuclei isolated from A172 cells treated with LiCl

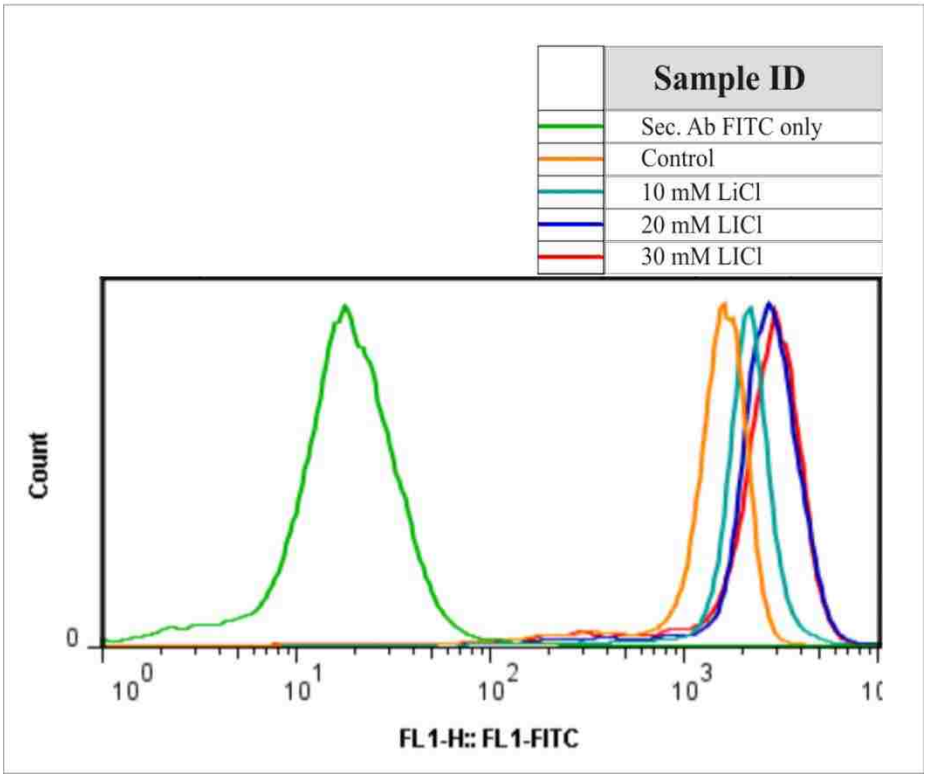


Fig. 19b Li^+ has a profound effect on the nuclear localization of β-catenin. A172 cells were treated with 0, 10, 20 or 30 mM LiCl for 24 hr. Stable nuclei were extracted from the Li^+ treated cells. The effect of Li^+ on the nuclear localization of β-catenin was assessed using a mouse mono clonal β-catenin primary antibody and an Alexa647 – conjugated secondary antibody. For each treatment, mean fluorescence per cell was determined from independent replicates and representative peak for each treatment is shown here. The x-axis values are represented in log scale.

Be^{+} treatment failed to induce clear nuclear translocation of β-catenin in A172, U87MG, Caki-1 and NIH/3T3 cells

The immunofluorescence data from NIH/3T3, A172 and A172 P1B cells indicates that beryllium is not able to induce prominent nuclear localization of β-Catenin. The immunofluorescence data was supported by the flow analysis of stable nuclei isolated from A172 cells after Be^{2+} and Li^{+} treatment. Traditionally it has been observed that majority of the work related to nuclear translocation of β-Catenin is based on immunofluorescence experiments. We thought it would be logical to analyze the nuclear localization of β-catenin using westerns. We decided to culture cells under the influence of appropriate concentration of GSK-3β inhibitors and then fractionate the cytoplasmic and nuclear proteins separately. A clean biochemically fractionated cytoplasmic and nuclear protein samples lacking cross contamination might work as a good additional system to analyze the nuclear localization of β-catenin. Be^{2+} treatment of A172, Caki-1 and NIH/3T3 cell lines for 24 hr caused an increase in the p53 levels, which is seen localized predominantly in the nuclear fraction. The up regulation of p53 in the Be^{2+} treated samples validates the success of inhibitor effect and acts as a positive control for beryllium induced up regulation of protein expression.

NIH/3T3 – 10 μM $BeSO_4$ has no effect on the p53 expression but 100 μM $BeSO_4$ leads to elevated levels of p53 (Fig. 20a, lane 6) as seen in the nuclear fraction. The β-catenin levels both in the cytoplasmic and nuclear fractions stay fairly constant indicating that probably Be^{2+} treatement failed to induce any detectable up regulation of β-catenin.

Caki-1 - 50 μM $BeSO_4$ treatment lead to elevated levels of p53 as observed in the cytoplasmic and nuclear fraction (Fig. 20b**,** lane 3&6**)**. A slight increase in the cytoplasmic levels of β-catenin in the presence of Be^{2+} is observed but the nuclear β-catenin levels stay fairly constant (b, lane

5&6) indicating that probably beryllium failed to induce detectable up regulation of β-catenin in the nuclear fractions of Caki-1 cells.

A172 - 10 μM $BeSO_4$ has no effect on the p53 upregulation but 100 μM $BeSO_4$ caused an increase in p53 levels as seen in the nuclear fraction and to an extent in the cytoplasmic fraction as well (Fig. 20c**,** lane 3&6**)**. The β-catenin level both in the cytoplasmic and nuclear fractions stays fairly constant (C, lane 2, 3 & 5, 6). 100 μM $CaCl_2$ was used as a negative control and was expected not induce any change either on p53 or β-catenin. These results indicate that probably beryllium failed to induce detectable up regulation of β-catenin in the nuclear fractions of A172 cells.

In U87MG the Be^{2+} induced p53 up regulation data of U87MG cells is not available but Be^{2+} treatment of U87MG cells causes up regulation of p53 as observed in Fig. 9. There is no effect of Be^{2+} treatment on the cytoplasmic and nuclear β-catenin levels in U87MG cells (Fig. 20d). 100 μM $CaCl_2$ was used as a negative control and was expected not induce any change either on p53 or β-catenin. It is possible that probably beryllium failed to induce detectable up regulation of β-catenin in the nuclear fractions of U87MG cells.

The strongest support for the minimal increase in the nuclear localization of β-catenin in the presence of beryllium comes from the immunofluorescence and flow analysis of stable nuclei experiments. These results suggest that probably Be^{2+} has minimal effect on the nuclear localization of β-catenin.

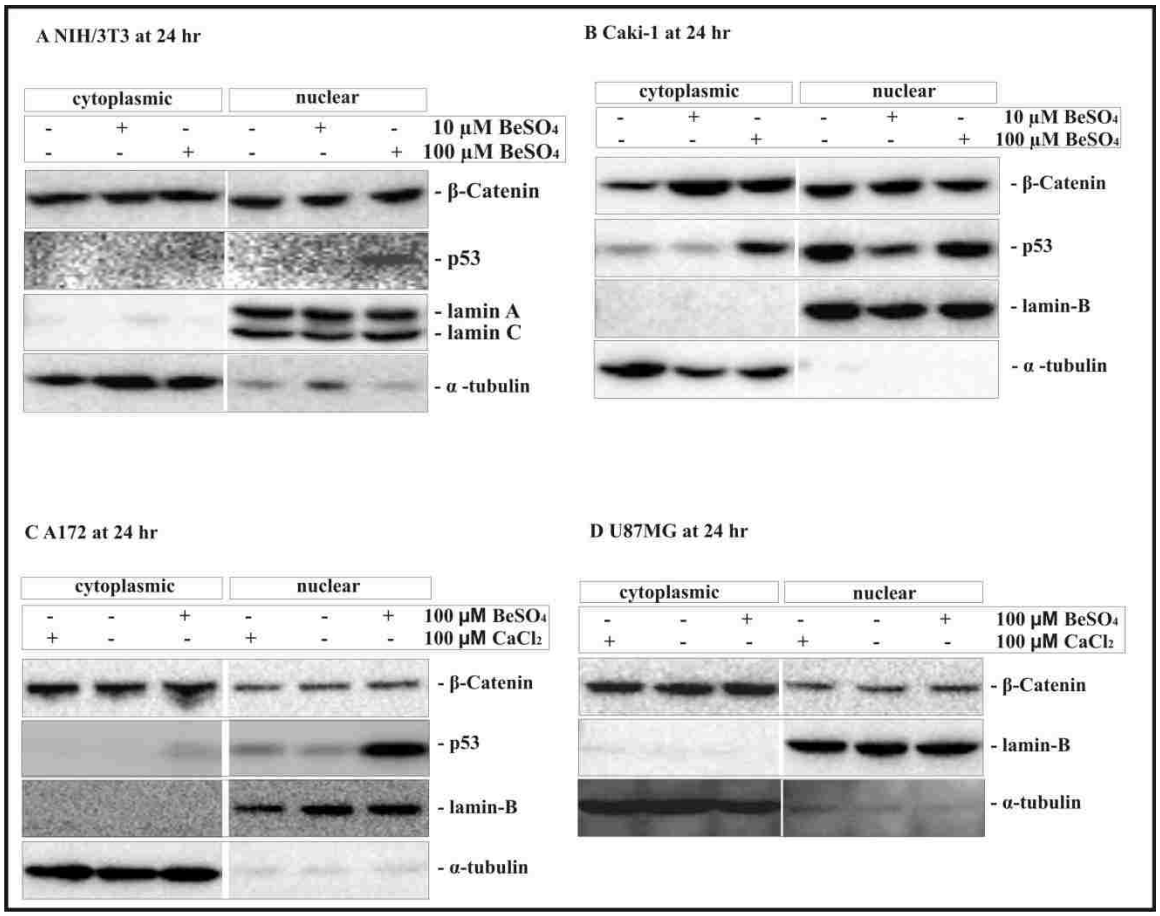


Fig. 20 Be^{2+} treatment seems to have minimal effect on the nuclear translocation of β-catenin. (a-b)NIH/3T3 and Caki-1 cells were treated with 0, 10 or 100 μM $BeSO_4$ for 24 hr and the cytoplasmic/nuclear proteins were fractionated. (c-d) A172 and U87MG cells were treated with 0, 100 μM $CaCl_2$ or 100 μM $BeSO_4$ for 24 hr and the cytoplasmic/nuclear proteins were extracted out separately as explained in the materials and methods section. The levels of total β-catenin, p53, lamin and tubulin were assessed by western blot analysis. The endogenous lamin and tubulin serve as loading control. The presence of tubulin or lamin in the cytoplasmic or nuclear fractions only shows the lack of cross contamination and the good quality of sample preparation

Beryllium has no effect on the stability of axin – a possible mechanism for the in ability of beryllium to induce nuclear localization of β-catenin

Beryllium treatment seems to be having minimal effect on the nuclear localization of β-catenin. Unlike Li^{+}, the inability of Be^{2+} to induce a major change in the nuclear localization of β-catenin is a surprising observation. In relation to beryllium's role as a GSK-3β inhibitor, two contrasting results are observed i.e. the lack of appreciable nuclear localization of β-catenin and decrease in the phosphorylation of glycogen synthase. These results indicate that Be^{2+} is able to inhibit the activity of GSK-3β fraction involved in the insulin signaling pathway. Contrarily it is observed that the GSK-3β enzyme fraction regulating the Wnt signaling pathway is fairly insulated from the inhibitory effects of Be^{2+}. Axin is an important substrate of GSK-3β and phosphorylation of axin by GSK-3β leads to its stabilization (Yamamoto et al., 1999). Axin, GSK-3β, APC (Adenomatous Polyposis Coli), casein kinase 1 (CK1), protein phosphatase 2A (PP2A) and E3-ubiquitin ligase β-TrCP are constituents of β-catenin destruction complex. Wnt ligands induced inhibition of GSK-3β is known to cause destabilization of axin (Willert et al., 1999). Inhibition of GSK-3β associated with the β-catenin destruction complex leads to dephosphorylation/destabilization of axin. The de phosphorylated form of axin cannot interact efficiently with β-catenin, which in turn leads to destabilization of the β-catenin destruction complex (Willert et. al., 1999). LiCl treatment caused a decrease in the levels of axin (Yamamoto et al., 1999) and it could be the possible reason behind the Li^{+} induced nuclear localization of β-catenin.

We hypothesized that the failure of beryllium treatment to induce any major changes in the nuclear localization of β-catenin could be due to its inability to perturb the stability of the β-catenin destruction complex. We investigated the effect of beryllium on the stability of axin - an important constituent of the β-catenin destruction complex.

In our study we demonstrated that Be^{2+} has no effect on the stability of axin in A172 and NIH/3T3 cells (Fig. 21a) Li^{+} caused a decrease in the total axin levels in A172 (human glioblastoma) (panel a, lane 7&8), however Be^{2+} seems to be having little effect on the stability of axin (panel a, lane 2-6). Fig. 21b Li^{+} caused a decrease in the total axin levels in NIH/3T3 cells (murine fibroblasts) (panel b, lane 3), however the total amount of axin in Be^{2+} treated sample is almost same as the control (panel 1, lane 2). Fig. 21c In NIH/3T3 (murine fibroblasts) beryllium at different concentrations did not induce any change in the total axin levels. These results indicate that the difference in the effect of Be^{2+} and Li^{+} on the nuclear localization of β-catenin could be because of their inability and ability respectively to disturb the β-catenin destruction complex respectively (**refer schematic diagram 4 on page 131**).

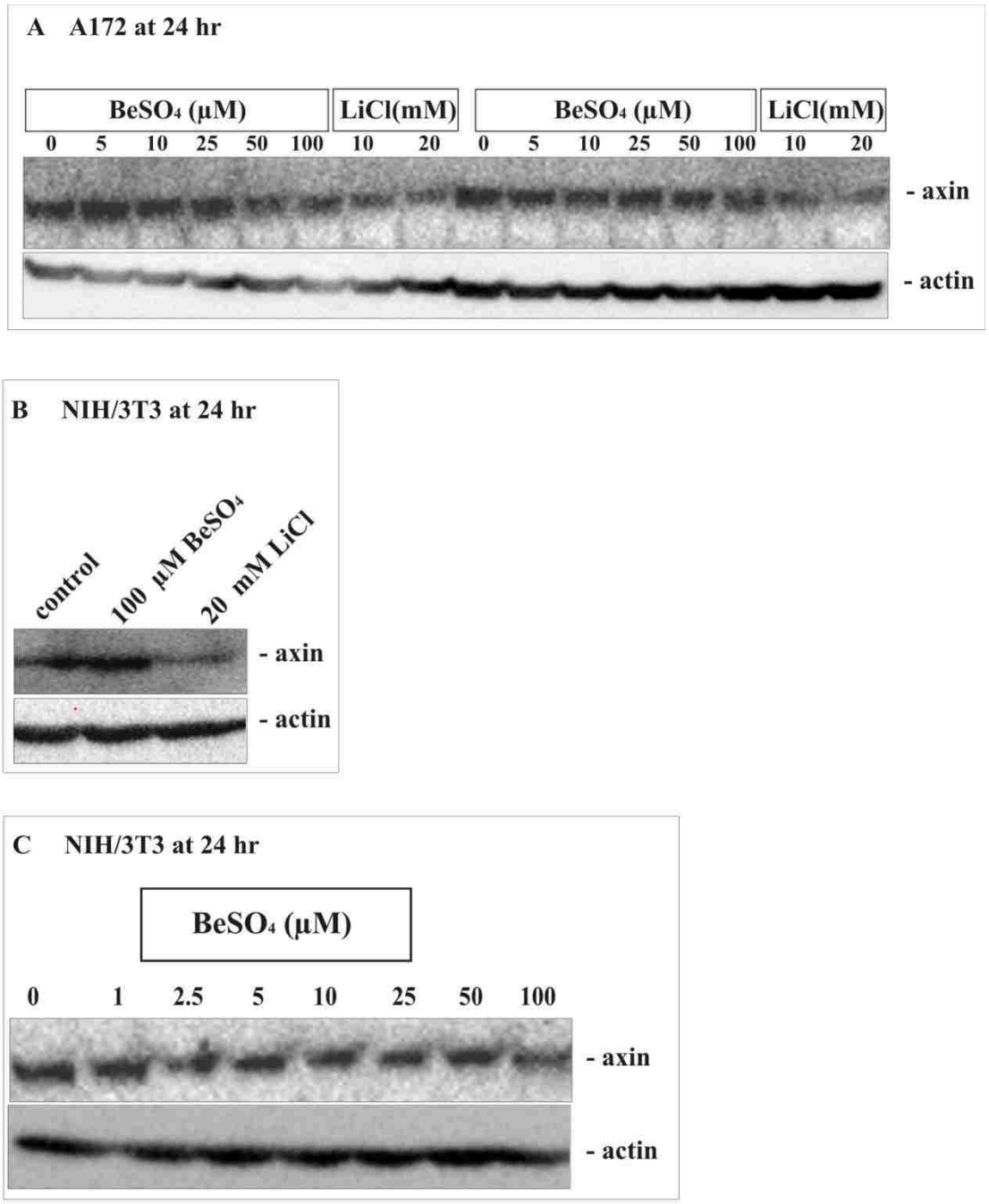
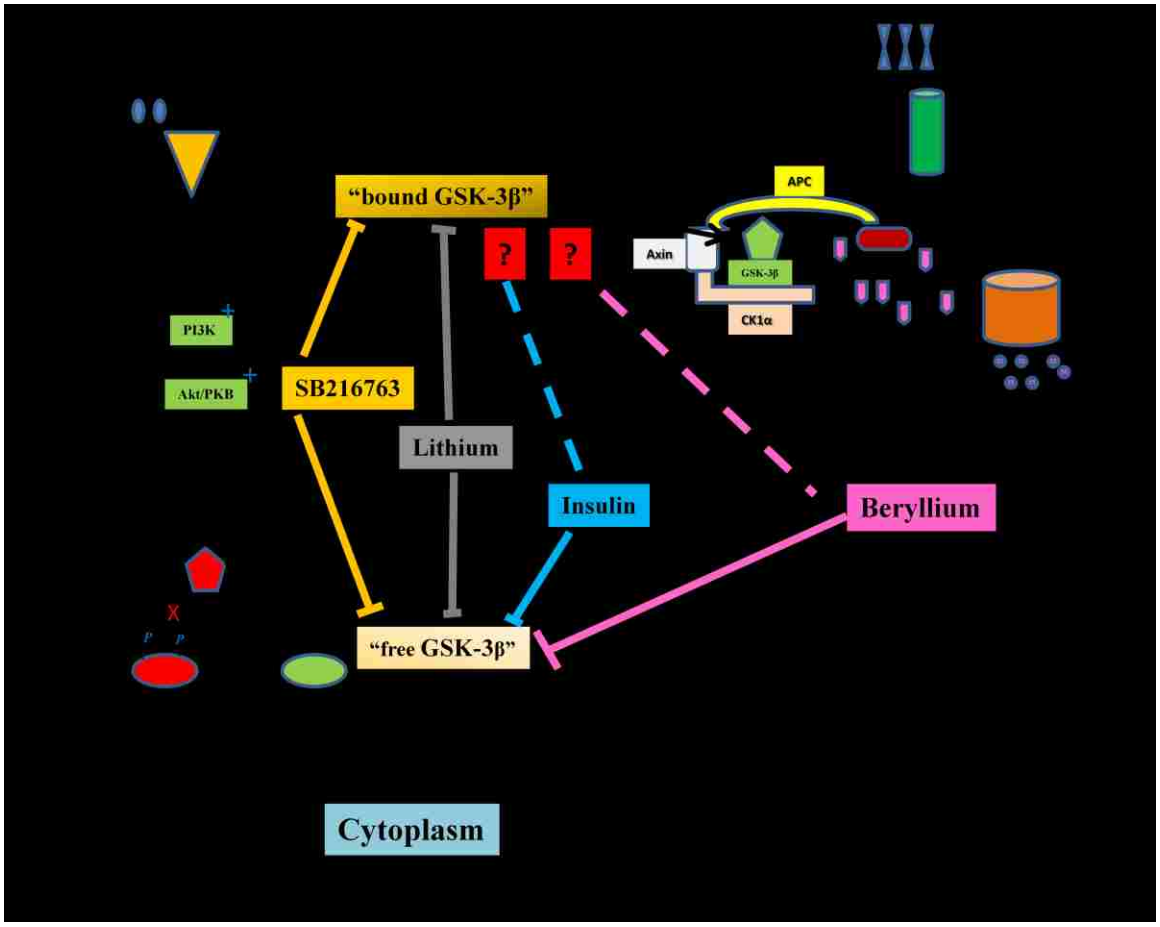


Fig. 21 Be^{2+} had no effect on the stability of the important β-catenin destruction complex constituent protein – axin. **a** A172 (human gliblastoma) treated with 0, 5, 10, 25, 50 or 100 μM $BeSO_4$ or 10 or 20 mM LiCl for 24 hr. **b** NIH/3T3 (murine fibroblasts) treated with 0, 100 μM $BeSO_4$ or 20 mM LiCl for 24 hr. **c** NIH/3T3 (murine fibroblasts) treated with 0, 1, 2.5, 5, 10, 25, 50 or 100 μM $BeSO_4$ for 24 hr. The effect of beryllium and lithium on the total axin levels was assessed using western blotting. The endogenous actin serving as loading control.

Schematic diagram comparing the GSK-3β inhibitory action of beryllium with other GSK-3β inhibitors



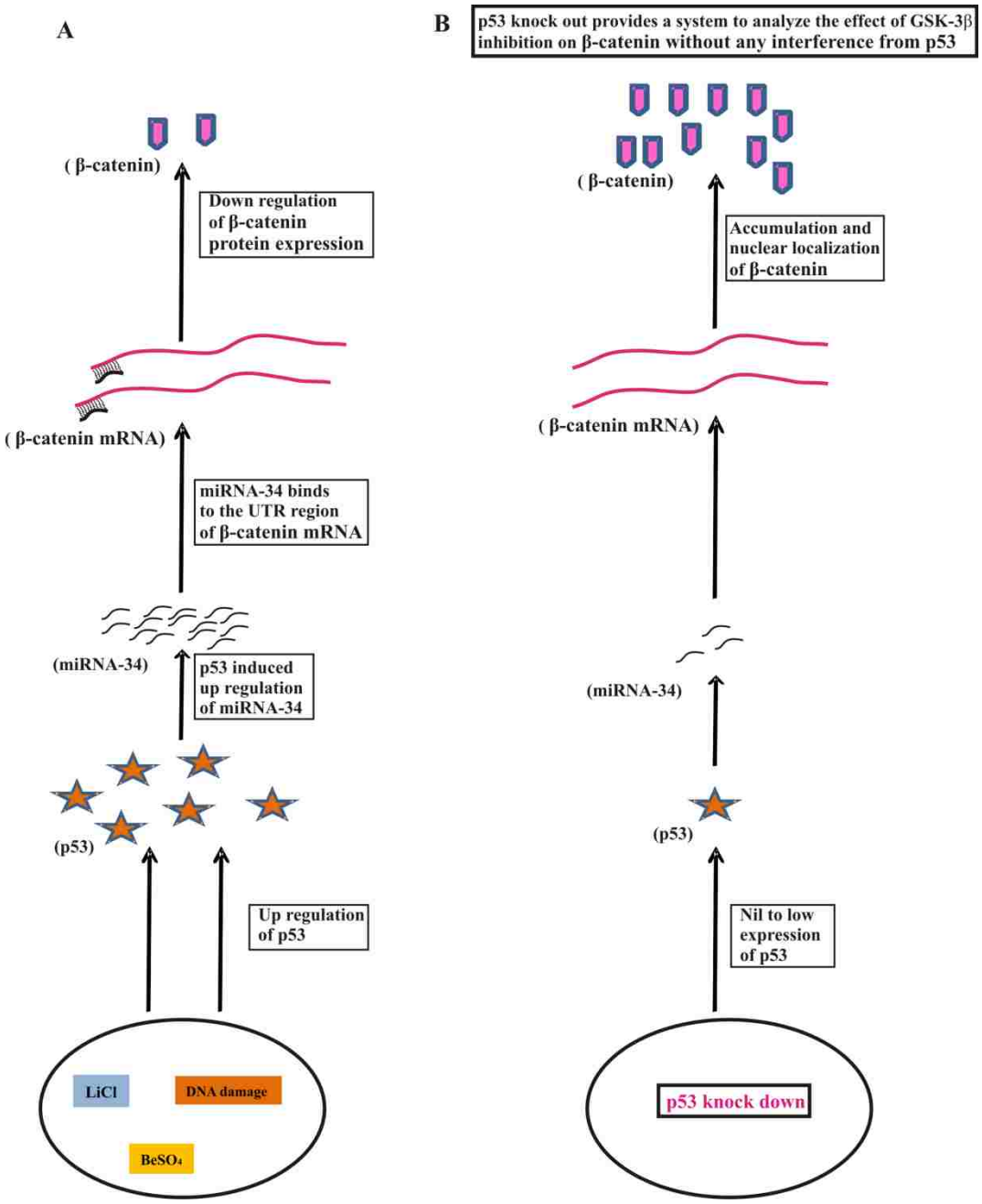
Schematic diagram 4: Differential regulation/inhibition of GSK-3β by beryllium. The inability of beryllium to destabilize axin indicates that probably Be^{2+} is not able to destabilize the β-catenin destruction complex. Hence beryllium may not be able to strongly inhibit the activity of "bound GSK-3β". However the other GSK-3β inhibitors like lithium and SB216763 seems to be inhgibiting both the fractions of GSK-3β. In this regard beryllium seems to be mimicking insulin because it has been demonstrated that insulin has no inhibitory effect towards the Wnt/β-catenin pathway (Ding et al., 2000).

Activation of p53 could be responsible for down regulating nuclear localization of β-catenin

The possibility of a cross talk between β-catenin and p53 in the progression of human cancers especially colorectal cancer has been suggested previously (Kinzler and Vogelstein, 1996). Later studies established a more direct interaction between p53 and β-catenin, wherein it was observed that deregulated β-catenin can lead to stabilization of p53 and vice versa (Damalas et al., 1999; Levina et al., 2004). The elevated p53 expression in response to various stimuli is an anti proliferative mechanism adopted by the human body for protection against the development of cancer (Oren, 2003; Oren et al., 2002). Deregulation of β-catenin has been implicated in the development of various cancers, pre dominantly colorectal cancer (Morin , 1999; Polakis, 2000; Patel et al.,, 2004; Wang et al., 2005; Thakur and Mishra, 2013). Elevated p53 elicits its anti proliferative effects in relation to β-catenin, firstly by blocking the pro-proliferative action of β-catenin and secondly by down regulating β-catenin expression (Sadot et al., 2001). (**refer schematic diagram 5 on next page**)

To eliminate the possibility of aberrant or mutant p53 interfering with the p53 mediated down regulation of β-catenin, cell lines expressing wt p53 were identified (Table 5).In our study we observed that A172, U87MG, Caki-1 and NIH/3T3 cells do not show any nuclear localization of β-catenin in the presence of Be^{2+} (Fig 20). Be^{2+} treatment caused up regulation of p53 expression (Lehnert et al., 2001; Coates et al., 2007; Gorjala and Gary, 2010) and it could be responsible for regulating the nuclear localization of β-catenin as explained in Sadot et al.

Schematic diagram explaining the role of p53 in regulating β-catenin



Schematic diagram 5: Upregulation of p53 leads to down regulation of β-catenin via miRNA-34. (Sadot et al., 2001; Cha et al., 2012)

Down regulation of p53 expression up regulates nuclear localization of β-catenin

We have used three different methods to "knock down" the p53 protein expression either post transcription or post translation.

p53 "knock down" using RNAi and its effect on the nuclear localization of β-catenin

Short interfering RNA (siRNA) is commercially available to induce an efficient p53 knock down. The p53 siRNA is a 20 nucleotide long synthetic double stranded RNA molecule, which will silence the p53 gene post transcriptionaly. Using siRNA to knock down p53 is the quickest and easiest way to obtain cells with down regulated p53 expression. The down regulation of p53 using siRNA is transient with the effect lasting until 48 – 72 hr. The p53 siRNA was transfected into A172 cells as explained in the materials and methods section. Post transfection A172 cells were allowed to grow in normal RPMI 1640 for 6 hr followed by treatment with $BeSO_4$ for 24 hr. A172 cells transfected with a non specific universal control siRNA served as the negative control. After Be^{2+} treatment the cytoplasmic/nuclear proteins were extracted out separately.

It was observed that in the presence of endogenous wt p53 there is no nuclear localization of β-catenin after Be^{2+} treatment (Fig. 20). Fig. 22 A prominent p53 band was observed in the nuclear samples isolated from A172 cells transfected with the universal control siRNA (Fig. 22, lane 4). The band corresponding to p53 disappears in the nuclear samples isolated from A172 cells transfected with p53 siRNA, indicating that p53 siRNA was successful in down regulating p53 expression (Fig. 22, lane 5). In A172 cells the successful knock down of p53 induced nuclear localization of β-catenin (lane 5) compared to the cells transfected with control siRNA (Fig. 22, lane 4). A more prominent increase in the nuclear β-catenin is observed when p53 knock down is coupled with Be^{2+} treatment (Fig. 22, lane 6).

The decrease of cytoplasmic β-catenin in the samples generated after p53 knockdown (panel 1, lane 2, 3) is complemented by the increase of nuclear β-catenin (panel 1, lane 5,6) indicating that the nuclear translocation of β-catenin could be induced by down regulation of p53.

This result demonstrates that, down regulation of p53 causes nuclear localization of β-catenin and the nuclear localization of β-catenin is amplified in the presence of Be^{2+}.

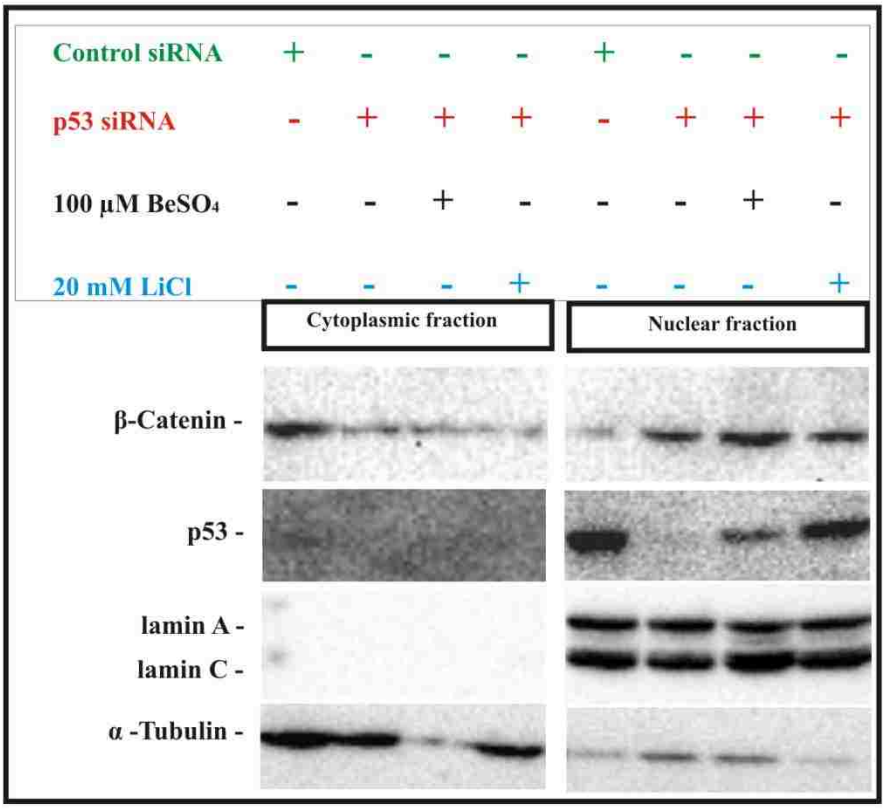


Fig. 22 Down regulation of p53 expression using RNAi causes nuclear localization of β-catenin. Cytoplasmic and nuclear proteins were extracted separately from A172 cells transfected with the control siRNA and p53 siRNA. The transfected cells were treated with 0 or 100 µM $BeSO_4$ for 24 hr. Western blots showing the protein levels of β-catenin, p53, lamin-A/C and tubulin. Lamin-A/C and tubulin acting as the loading control.

p53 knock down using shRNA – Lentiviral vector system

Short hairpin RNA (shRNA) is a sequence inserted into a lentivirus vector plasmid. Successful insertion of the shRNA sequence into the genome and its subsequent expression will induce silencing of the target gene expression. The advantage of this method is the development of stable cell lines with p53 knock down.

The successful transfection of the lentivirus vector plasmid and down regulation of p53 protein via the shRNA was confirmed using western blotting (Fig. 23). A172 cells were transfected with the p53 shRNA Lentivirus vector plasmid (as explained in the materials and methods section). As a negative control A172 cells were transfected with an empty Lentivirus vector plasmid called as A172 p53 control. Successful transfection and expression of the p53 shRNA induced a down regulation of p53 expression (Fig. 23, lane 2). The p53 expression is not disturbed in the A172 p53 control cells and the p53 levels are comparable to the untransfected A172 cells (Fig. 23, lane 1, 3). Be^{2+} treatment at 10 μM for 24 hr did not induce any increase in the p53 protein levels in the p53 KO cells (Fig. 23, lane 4). Conversely a Be^{2+} induced increase in p53 expression is observed in the untransfected and p53 control A172 cells (Fig. 23, lane 4, 6). This experiment validates the successful down regulation of p53 expression by the shRNA lenti virus vector system.

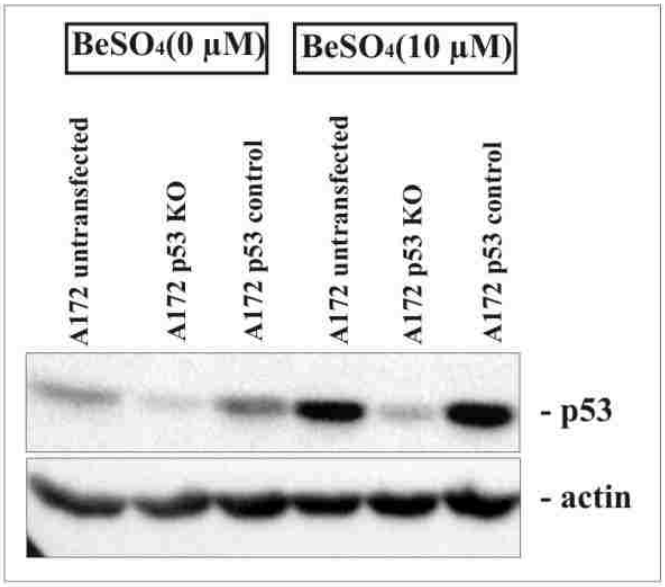


Fig. 23 Down regulation of p53 by the shRNA lentivirus vector. The untransfected A172 cells, A172 p53 KO and A172 p53 control cells were treated with 0 or 10 µM $BeSO_4$ for 24 hr. Total cell lysates were analyzed for p53 and actin after the Be^{2+} treatment using western blotting.

p53 knock down using shRNA and its effect on the nuclear localization of β-catenin

The A172 cells carrying the lentivirus vector plasmid were designated as A172 p53 KO cells (Fig. 24). The cytoplasmic and nuclear protein fractions isolated from A172 p53 KO treated with $BeSO_4$ show nuclear accumulation of β-catenin (Fig. 24, lane 5&6). The nuclear localization of β-catenin in $BeSO_4$ treated A172 p53 KO is quite evident in comparison to the control (untreated) A172 p53 KO (Fig. 24, lane 4**).**

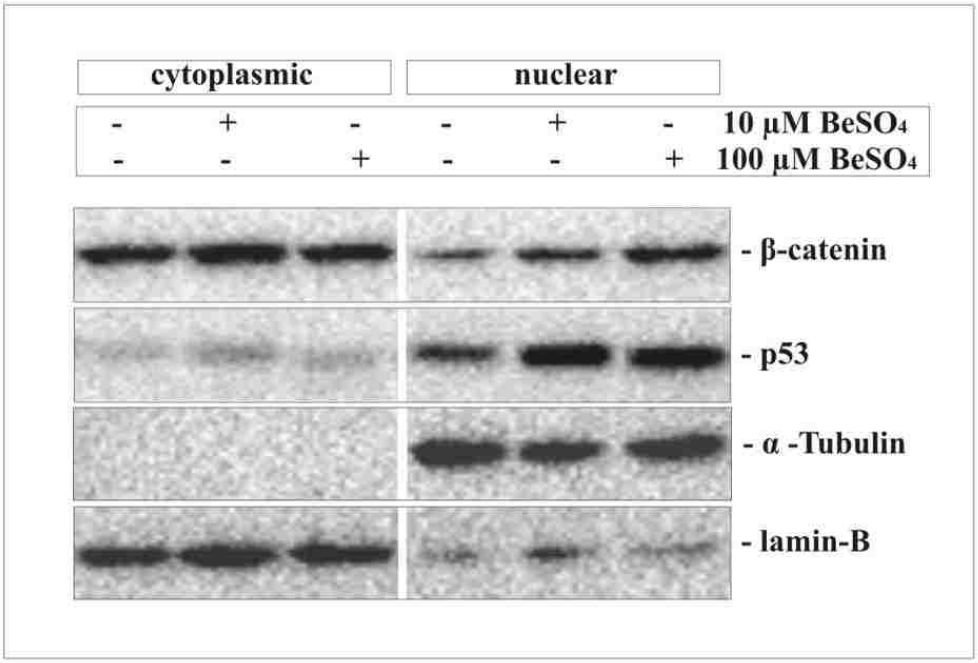


Fig. 24 Down regulation of p53 protein via the shRNA lentivirus vector aids Be^{2+} in inducing nuclear localization of β-catenin. Cytoplasmic and nuclear proteins were extracted separately from A172 p53 KO cell treated with 0, 10 or 100 μM $BeSO_4$ for 24 hr. Western blots showing the protein levels of β-catenin, p53, lamin-B and tubulin. Be^{2+} induced nuclear localization of β-catenin is observed in the nuclear samples (panel 1, lane 5, 6). Lamin-B and tubulin acting as the loading control.

Down regulation of endogenous p53 using the HPV E6 protein

The down regulation of endogenous p53 using the HPV gene 6 product provides a good system to analyze the cellular effects elicited by down regulation of p53. A172 and U87MG cells transfected with pCMV-E6 plasmid and expressing the human papillomavirus 16 E6 gene were obtained as a gift from Dr. Cairncross, University of Calgary. The HPV E6 genes can be transfected into mammalian cells to create cell lines with down regulated p53 expression (Xu et al., 2005). The successful translation of HPV E6 genes leads to synthesis of the E6 protein, which can bind to wt p53 and induce ubiquitin mediated degradation of wt p53 (Werness et al., 1990; Crook et al., 1991). The cell lines expressing HPV E6 protein known as the A172 E6 and U87MG E6 have down regulated p53 expression as explained in Xu et al (2005). A172 E6 and U87MG E6 cells were treated with $BeSO_4$ and the effect of p53 down regulation on β-catenin was analyzed in the presence of Be^{2+}.

The total cell lysates of U87MG E6 show minimal p53 expression and a subsequent increase in the total β-catenin is observed in a concentration dependent manner. Compared to the cells treated with 0 or 10 μM $BeSO_4$ (Fig. 25**,** lane-1&2) a marked increase in the total β-catenin amount is observed in the U87MG E6 cells treated with 30 or 100 μM $BeSO_4$ (lane-3&4).

The total cells lysates of Be^{2+} treated A172 E6 cells show p53 bands because in E6 cell lines the p53 expression is not knocked out completely (Fig. 26). The total β-catenin levels in A172 E6 cells are almost constant in the presence of increasing concentrations of $BeSO_4$.

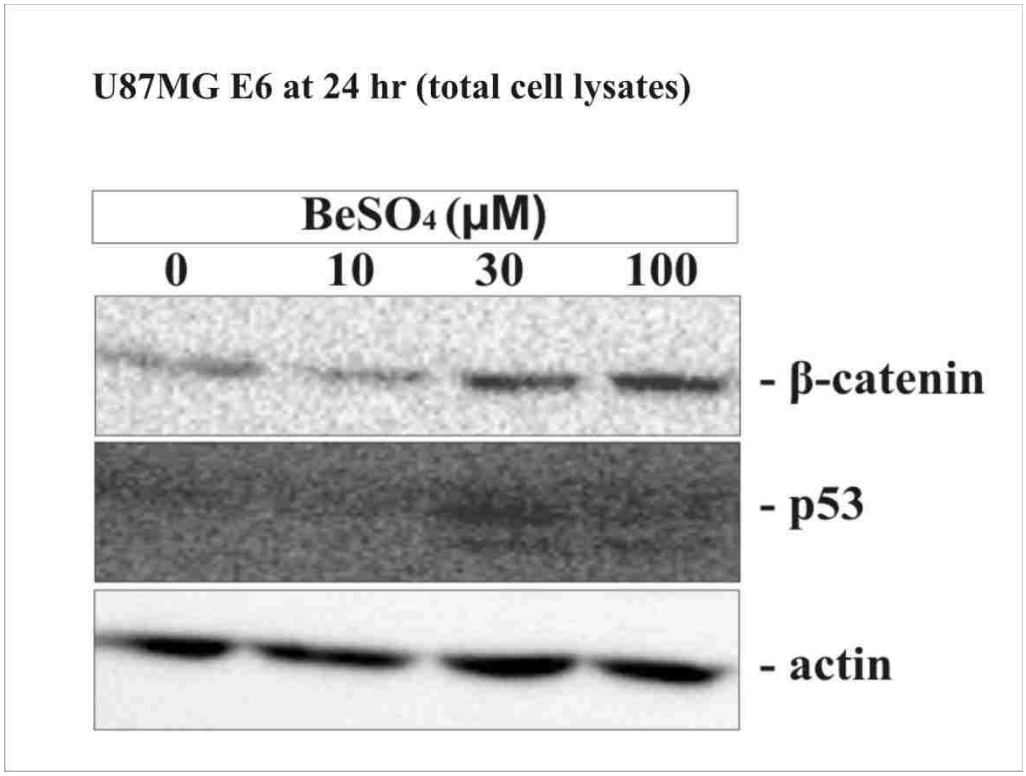


Fig. 25 Effect of down regulated p53 on the β-catenin levels in total cell lysates of U87MG E6. U87MG E6 were treated with 0, 10, 30 or 100 μM with $BeSO_4$ for 24 hr. After total cell lysate extraction from the Be^{2+} treated cells, western blotting was used to analyze the protein levels of β-catenin, p53 with actin acting as the loading control.

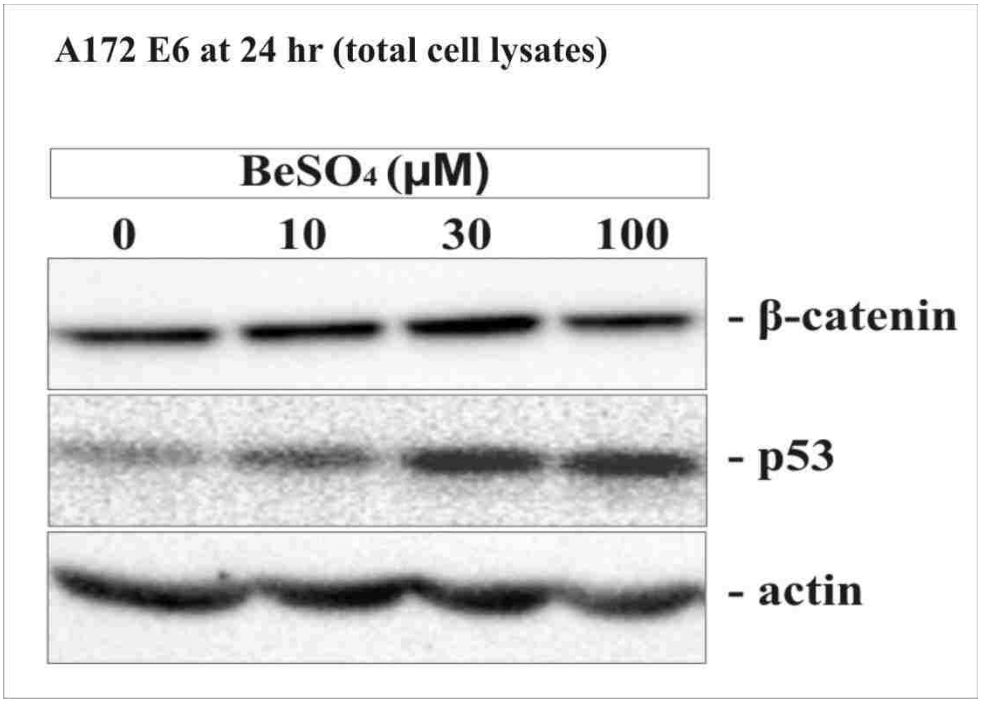


Fig. 26 Effect of down regulated p53 on the β-catenin levels in total cell lysates of A172 E6. A172 E6 were treated with 0, 10, 30 or 100 μM with $BeSO_4$ for 24 hr. After total cell lysate extraction from the Be^{2+} treated cells, western blotting was used to analyze the protein levels of β-catenin, p53 with actin acting as the loading control.

In order to analyze the effect of p53 down regulation on the nuclear localization of β-catenin A172 E6 and U87MG E6 cells were treated with $BeSO_4$ for 24 hr. The cytoplasmic and nuclear proteins were fractionated out separately.

Be^{2+} treatment does not seem to be having any effect on the expression/up regulation of p53 in U87MG E6 cells. Be^{2+} treatment at 30 µM or 100 µM concentration leads to nuclear accumulation of β-catenin (Fig. 27, lane 5-6) and increase in the cytoplasmic levels of β-catenin is also observed (lane 2, 3).

In A172 E6 cells, Be^{2+} treatment caused an optimum increase in the p53 expression (Fig. 28, lane 2, 3 or 5, 6). An increase in the nuclear β-catenin is observed in the nuclear fraction of the A172 E6 treated with 100 µM $BeSO_4$ (lane 6).

The analysis of the β-catenin accumulation/localization in the E6 cell lines shows that down regulation of p53 leads to stabilization and nuclear localization of β-catenin.

The down regulation of p53 expression was achieved by three independent techniques "p53 RNAi knockdown", "shRNA Lentivirus vector" and "E6 cell lines expressing HPV E6 protein". The results from these three independent experiments demonstrate that down regulation of p53 leads to nuclear localization of β-catenin. It is also observed that the nuclear localization of β-catenin induced by the down regulation of p53 is amplified in the presence of Be^{2+}.

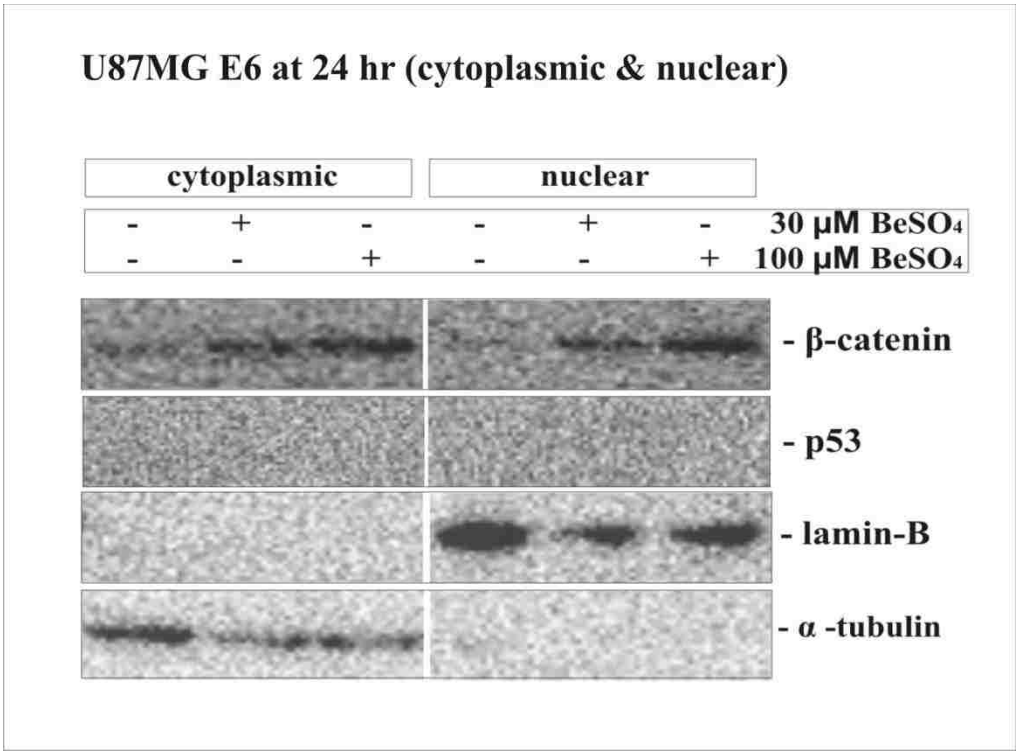


Fig. 27 Effect of down regulated p53 on the nuclear localization of β-catenin in U87MG E6. U87MG E6 cells were treated with 0, 30 or 100 μM with $BeSO_4$ for 24 hr. The cytoplasmic/nuclear proteins were extracted out separately from the Be^{2+} treated cells, western blotting was used to analyze the protein levels of β-catenin and p53. Tubulin and lamin acting as the loading controls.

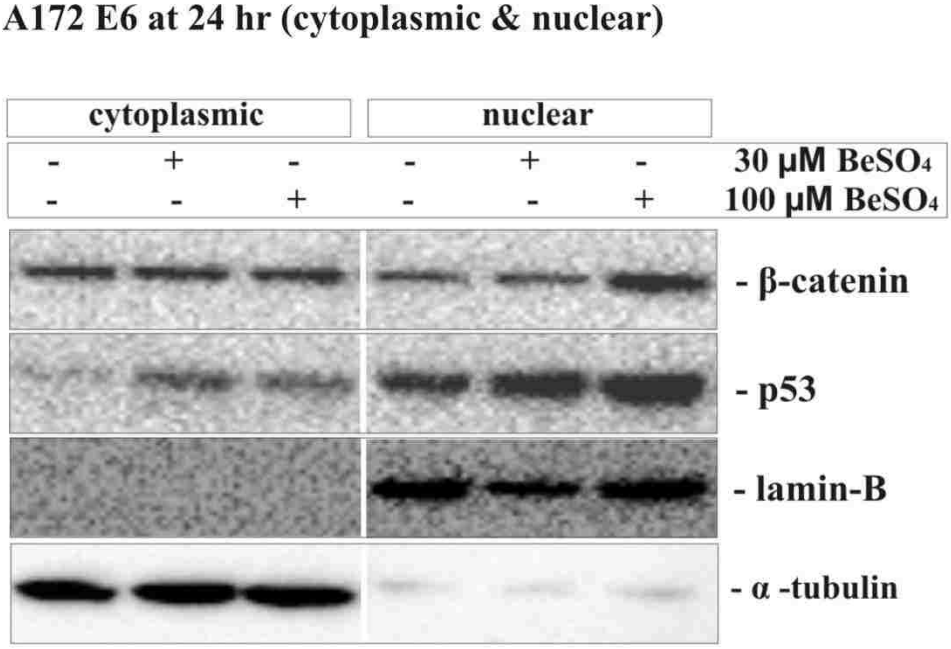


Fig. 28 Effect of down regulated p53 on the nuclear localization of β-catenin in A172 E6. A172 E6 cells were treated with 0, 30 or 100 μM with $BeSO_4$ for 24 hr. The cytoplasmic/nuclear proteins were extracted out separately from the Be^{2+} treated cells, western blotting was used to analyze the protein levels of β-catenin and p53. Tubulin and lamin acting as the loading controls

Beryllium and lithium have contrasting effects on the phosphorylation status of β-catenin

β-catenin is phosphorylated on Ser-33, Ser–37 and Thr-41 by GSK-3β (Ikeda et al., 1998; Sadot et al., 2002; Sutherland, 2011). The phosphorylated β-catenin is then ubiquitinated followed by proteasomal degradation. As an aftermath of Li^+ mediated GSK-3β inhibition, a decrease in phospho β-catenin level is observed in 293T cells (Sadot et al., 2002). The assessment of decrease/increase in the phospho β-catenin levels, in the presence of GSK-3β inhibitors can act as a direct and indisputable method of establishing the effect of GSK-3β inhibitors on Wnt/β-catenin signaling pathway.

We investigated the effect of Be^{2+} treatment on the phospho β-catenin levels in A172 cells. Be^{2+} treatment did not induce any increase or decrease in the phospho β-catenin level (Fig. 29**,** lane 2, 3, 4, 5&6) and correspondingly the total β-catenin levels too stayed fairly constant (lane 2, 3, 4, 5&6). These results suggest that probably Be^{2+} has nil to minimal inhibitory effect on the Wnt signaling pathway. The analysis of the phospho β-catenin level in Li^+ treated samples showed that Li^+ induces an increase in the phospho β-catenin (lane 7&8), which was unexpected. The lack of increase in the total β-catenin levels indicates that Li^+ treatment is primarily modulating the phosphorylation status of β-catenin only (lane 7, 8). The increase in the p53 protein levels in the presence of $BeSO_4$ and LiCl validates the effectiveness of Be^{2+} and Li^+ treatment on A172 cells.

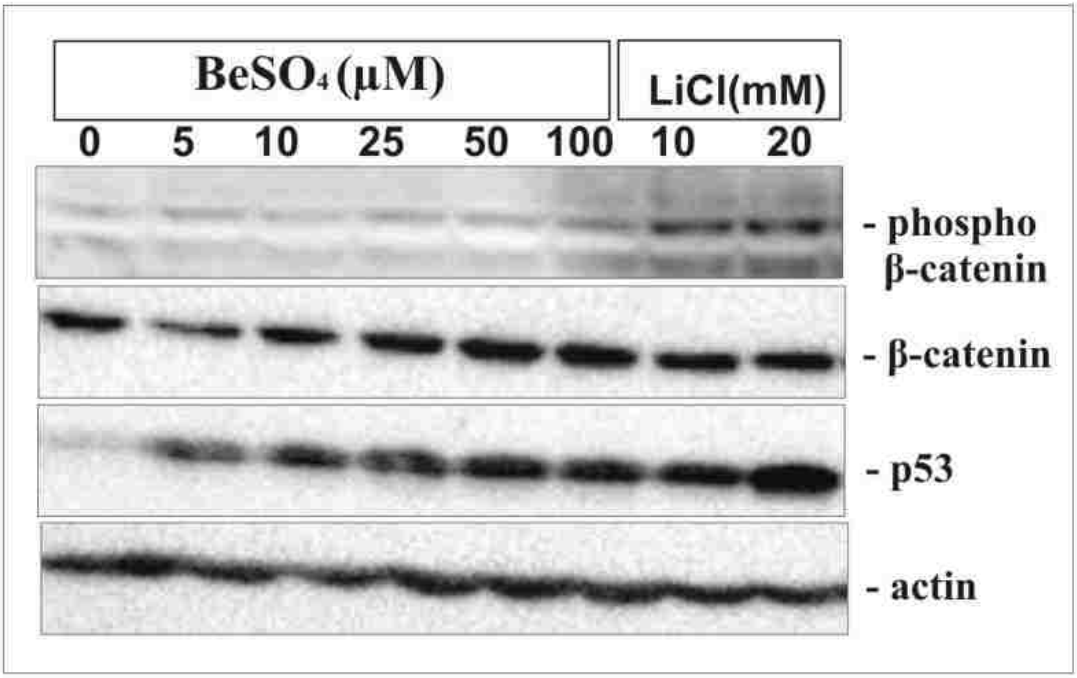


Fig. 29 Be^{2+} has no effect on the phosphorylation status of β-catenin. A172 cells were treated with 0, 5, 10, 25, 50 or 100 μM $BeSO_4$ or 0, 10 or 20 mM LiCl for 24 hr. The phospho β-catenin (Ser–33), total β-catenin, p53 and actin protein levels were assessed using western blotting with actin serving as the loading control.

MG132 - a proteasome inhibitor, leads to accumulation of phospho β-catenin in A172 cells

MG132 (sc-201270) is a strong proteasome inhibitor (Lee and Goldberg, 1998). Inhibition of the proteasome machinery using MG132 should have a positive effect on β-catenin turn over. A172 cells were treated with 100 µM $BeSO_4$ or 20 mM LiCl for 18 hr and then the Be^{2+}- Li^{+} RPMI was replaced with Be^{2+}- Li^{+} RPMI supplemented with 10 µM MG132 and the cells were cultured under the effect of MG132 for 6 hr.

In the presence of MG132 a slight increase in the phospho β-catenin was observed in the control samples (Fig. 30, lane 4) compared to the control samples lacking MG132 (lane 1). A profound increase in the p53 protein level is observed in the MG132 control cells (lane 4). The increase in p53 protein levels in the MG132 samples proves the effective inhibitory effect of MG132 on proteasome machinery. In the absence of MG132 the phospho β-catenin levels of the Be^{2+} treated samples are comparable to the control phospho β-catenin level (lane 1&2). In the presence of MG132 as well the phospho β-catenin levels of the Be^{2+} treated samples are comparable to the control phospho β-catenin level (lane 4&5). These results indicate that may be Be^{2+} has no role in inducing the increase in phospho β-catenin in the MG132 + Be^{2+} treated samples. The slight increase in the phospho β-catenin levels in the MG132 + Be^{2+} treated samples compared to the Be^{2+} treated samples could be because of the inhibitory effect of MG132 on the proteasome machinery (lane 2&4). The phospho β-catenin levels of the Li^{+} treated samples both in the presence and absence of MG132 are almost the same (lane 3&6). It indicates that probably Li^{+}-induced increase in the phospho β-catenin is an intrinsic property of Li^{+}, which could be independent of the effects of MG132.

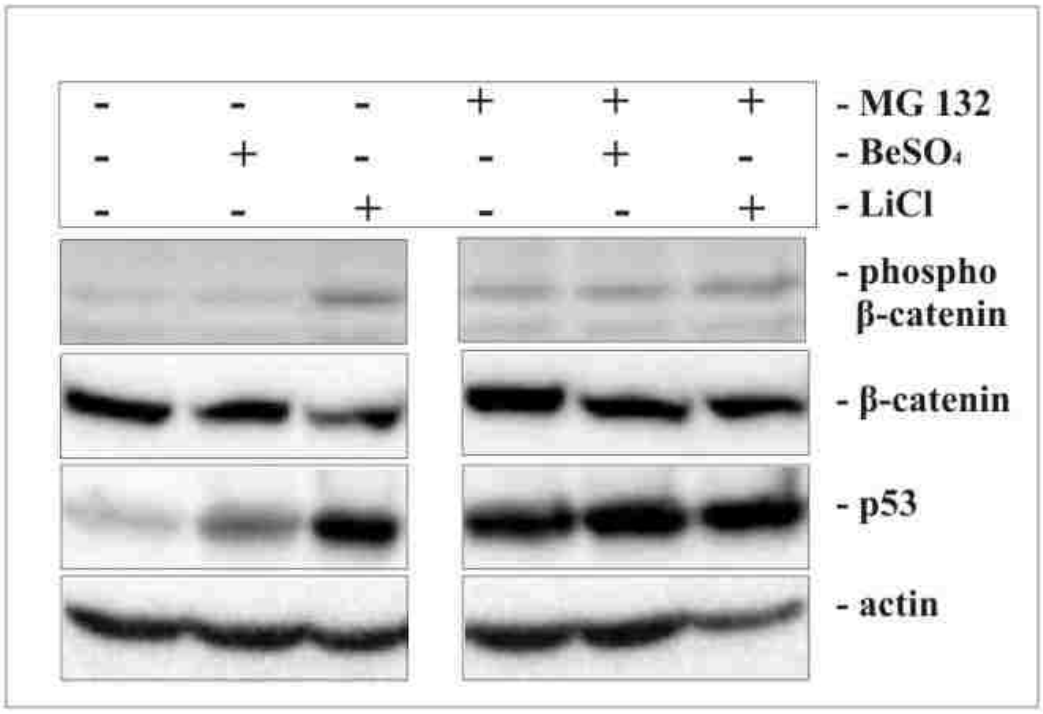


Fig. 30 Proteasome inhibitor (MG132) leads to an increase in the phospho β-catenin level in Be^{2+} treated samples but not in the Li^{+} samples. Western blots showing the protein levels of phospho β-catenin (Ser -33), total β-catenin, p53 and actin. A172 cells were cultured either in the absence (Fig 30 left) or presence (Fig 30 right) of 10 μM MG132. A172 cells were treated with 0, 100 μM Be SO_4 or 20 mM LiCl for 24 hr.

Down regulation of p53 has no effect on phospho-β-catenin

The observation that Li^+ caused an increase in phospho β-catenin in A172 cells was unexpected, because β-catenin is a substrate for phosphorylation by GSK-3β and Li^+ acts as an inhibitor of GSK-3β kinase activity. Moreover, Sadot et al. observed that Li^+ causes a decrease in phospho-β-catenin when 293T cells are used (Sadot et al., 2002). The A172 cells shown in Fig. 29 and 30 have wild type p53. 293T cells are human embryonic kidney cells that have been transformed with SV40 large T antigen, which inactivates p53. Considering the potential for complicated interactions between p53 signaling and the Wnt/β-catenin network, we wondered whether p53 status could account for the divergent effects of lithium on phospho-β-catenin when comparing our results to those of Sadot et al. Therefore, we repeated this experiment using the p53 shRNA-expressing A172 cells (p53 KO A172 cells) (Fig. 31). In p53 KO A172 the p53 expression is down regulated (Fig. 23). However, our results with p53 KO A172 reproduced our earlier results when A172 cells expressing wild type p53 were used: In p53 KO A172 cells as well Li^+ caused an increase in the phospho-β-catenin but beryllium did not at 24 hr. These results were reproduced after 48 hr treatment time as well. Our results from p53 KO A172 cells (Fig. 31) indicate that probably p53 has no role to play in the lithium induced increase of phospho β-catenin.

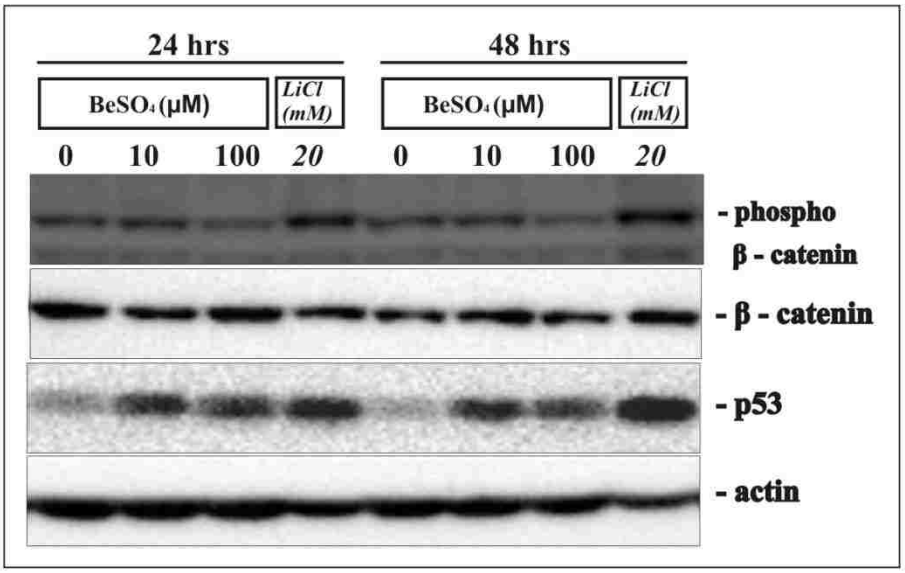


Fig. 31 Down regulation of p53 has no effect on the phospho-β-catenin levels. A172 p53 KO cells were treated with 0 , 10 or 100 μM $BeSO_4$ or 20 mM LiCl for 24 or 48 hr. Western blots showing the protein levels of phospho β-catenin (Ser -33), total β-catenin, p53 and actin.

Beryllium in combination with MG132 (a proteasome inhibitor) induces an increase in the phospho-β-catenin levels in A172 p53 KO cells

In the presence of a proteasome inhibitor an increase in the phosphor-β-catenin levels was observed (Fig. 30). We wondered what would be the effect of MG132 on shRNA-expressing p53 KO A172 cells. A172 cells were treated with 100 μM $BeSO_4$ or 20 mM LiCl for 18 hr and then the Be^{2+}-Li^{+} RPMI was replaced with Be^{2+}-Li^{+} RPMI supplemented with 10 μM MG132 and the cells were cultured under the effect of MG132 for 6 hr.

In p53 KO A172 cells the use of MG132 caused phospho β-catenin to accumulate at higher levels(Fig. 32). There is a marked increase in the phospho β-catenin levels in the untreated (MG132) samples compared to the untreated samples lacking the proteasome inhibitor MG132. The beryllium treated samples seems to be following the pattern of untreated cells in terms of phospho β-catenin accumulation (appreciable amount of phospho-β-catenin accumulation in the presence of proteasome inhibitor). The p53 KO A172 cells seems to be imitating the normal A172 cells in showing a little difference in the phospho β-catenin levels in the presence or absence of proteasome inhibitor. The results from A172 cells (Fig. 30) and p53 KO A172 cells (Fig. 32) indicate and reiterate the point that probably p53 has no role in the lithium induced increase of phospho β-catenin .

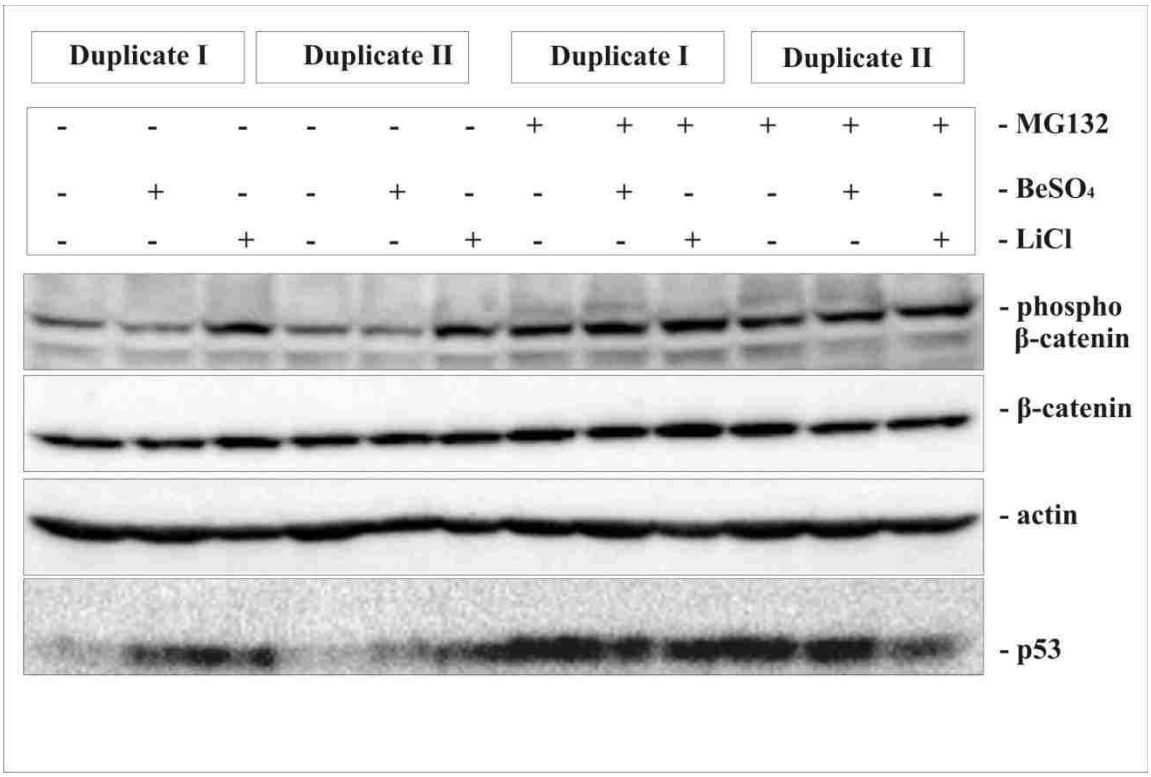


Fig. 32 Down regulation of p53 coupled with proteasome inhibiton causes accumulation of phospho-β-catenin in p53 KO A172 cells. p53 KO A172 cells were treated with 0 or 100 μM $BeSO_4$ or 20 mM LiCl for 24 hr. Western blots showing the protein levels of phospho β-catenin (Ser -33), total β-catenin, p53 and actin. p53 KO A172 cells were cultured either in the absence or presence of 10 μM MG132.

Discussion

In our previous study we established that Be^{2+} is a potent GSK-3β inhibitor and it doesn't elicits any increase in the Ser-9 phosphorylation of GSK-3β in A172 and HFL cells (Mudireddy et al., 2014). Analysis of the Ser-9 phosphorylation of GSK-3β in NIH/3T3 and Caki-1 cells treated with $BeSO_4$ for 24 hr shows that Be^{2+} treatment caused an increase in Ser-9 phosphorylation of GSK-3β (Fig. 9 & 10). The ability of Be^{2+} to induce Ser-9 phosphorylation of GSK-3β seems to be a cell type specific action. In our present study Be^{2+} did not elicit any change in the Ser-9 phosphorylation status of GSK-3β in A172 and U87MG cells (Fig. 8&9). Surprisingly Li^{+} too did not induce any change in the Ser-9 phosphorylation of GSK-3β in U87MG cells; this is in contrast to the results reported in Atkins et al (2012). (Fig. 9b, lane 4). The comparison of beryllium's effect on the Ser-9 phosphorylation of GSK-3β with other GSK-3β inhibitors like Li^{+}, SB216763, insulin or rottlerin shows that it is only Be^{2+} which elicits a cell type specific response in A172, NIH/3T3 and Caki-1 cells (Fig. 12 & Table 5). Li^{+} influences the GSK-3β to TIMAP to PP1 positive feedback loop eliciting an increase in the Ser-9 phosphorylation of GSK-3β (Zhang et al., 2003). It is possible that in some cell types Be^{2+} may not be able to regulate the GSK-3β to TIMAP to PP1 feedback loop because of which no increase in the Ser-9 phosphorylation of GSK-3β is observed. The uniqueness of beryllium's role as a GSK-3β inhibitor lies in its ability to regulate Ser-9 phosphorylation of GSK-3β differentially depending upon the specific cell lines. In our comparative study here Be^{2+} is the only GSK-3β inhibitor among Li^{+}, SB216763 and insulin, which has a cell type specific effect on the Ser-9 phosphorylation of GSK-3β.

Be^{2+} caused a decrease in the phosphorylation of glycogen synthase (GS) in NIH/3T3 cells (Fig. 15&16). This is the first time ever the inhibitory effect of Be^{2+} on GSK-3β is demonstrated by assessing the effect of Be^{2+} on the endogenous target proteins that are downstream of GSK-3β. Be^{2+} caused an increase in the Ser-9 phosphorylation of GSK-3β and it could lead to its

inactivation in NIH/3T3 cells. The increase in the Ser-9 phosphorylation of GSK-3β in the Be^{2+} treated samples is complemented well by the decrease in phosphorylation of glycogen synthase. It has been demonstrated that Li^{+} causes a decrease in pGS levels in L6 muscle cells and 3T3-L1 adipocytes (MacAualy et al., 2003). An intriguing aspect related to the effect of GSK-3β inhibitors on the phosphorylation status of glycogen synthase has been the selective dephosphorylation of glycogen synthase isoform-1 only. The expression of glycogen synthase isoforms is tissue specific (Browner et al., 1989; Nuttall et al., 1994). It has been reported that unicellular organisms like synechocystis (cyanobacteria) and yeast express both the isoforms of glycogen synthase (Frakas et al., 1991; Yoo et al., 2014). As explained in the results section NIH/3T3 could possibly be expressing both the isoforms of glycogen synthase. It is not clear why lithium, beryllium or SB216763 are able to induce a decrease in the phosphorylation status of glycogen synthase-1 only. The regulation of glycogen synthase is a complicated phenomenon which is not clearly understood yet. It has been reported that in yeast the expression of the glycogen synthase isoforms is differentially regulated (Frakas et al., 1991). Phosphorylation and dephosphorylation of glycogen synthase plays an important role in its regulation. It could be possible that the phosphorylation status of glycogen synthase could be differentially regulated as well. It has been observed that kinases are closely associated with phosphatases and they work in tandem in regulating the phosphorylation status of substrates. A good example of a kinase/phosphatase pair working in tandem would be the regulation of Ser-9 phosphorylation of GSK-3β as explained in the Zhang's model (schematic diagram 3). Along with different kinases certain phosphatases like PP1G has been implicated in regulating the phosphorylation status of glycogen synthase (Smith et al., 1983; Aschenbach et al., 2001). There is a possibility that there might be a phosphatase that is specific for the isoforms of glycogen synthase and the GSK-3β inhibitors could be interfering with its phosphatase activity. However this is a speculation and there is no information to support such a possibility.

Dominant phosphorylation sites: GSK-3β is one of the many kinases that phosphorylate glycogen synthase and regulate its activity (explained in Roach et al., 2012). It has been proposed that in rabbit muscle cells expressing glycogen synthase-1 the phosphorylation of site 2 (*Ser-8), site 2a (*Ser-11), site 3a (*Ser-641) and site 3b (*Ser-645) play an important role in its regulation (* amino acid numbers in relation to mouse glycogen synthase) (Skurat et al., 1994, 1995). The phospho-GS antibody used in this study is specific to the pSer-641 and pSer-645 residues. In liver glycogen synthase isoform 2 is present and the dominant phosphorylation site on glycogen synthase-2 is site 2 (*Ser-8) (Ros et al., 2009). It could be possible that the phosphorylation status of Ser-641 and Ser-645 might be playing a less important role in the regulation of glycogen synthase-2. Hence it can be speculated that the use of GSK-3β inhibitors in NIH/3T3 cells seems to have no effect on the phosphorylation status of glycogen synthase-2 (Fig 16). However the effect of GSK-3β inhibitors on the pGS levels has to be investigated in a cell line representing hepatic lineage. In this way the precise effect of GSK-3β inhibitors on the phosphorylation status of glycogen synthase-2 can be analyzed.

Compartmentalization of GS: Prats et al showed that along with the phosphorylation/dephosphorylation mechanism compartmentalization of glycogen synthase seems to be playing an important role in its regulation (Prats et al., 2009). It could be possible that the glycogen synthase isoforms in the NIH/3T3 cells could be compartmentalized and hence differentially regulated by GSK-3β inhibitors.

Li^+ mimics the actions of insulin and inhibits GSK-3β, which in turn has a positive effect on glycogen metabolism (Cheng et al., 1983; Bosch et al., 1986; Choi et al., 2000).Whether the Be^{2+} treatment induced dephosphorylation of glycogen synthase (GS) has a positive effect on the activity of GS and glycogen metabolism has to be investigated further.

The most common way to demonstrate the after effects of GSK-3β inhibition is by assessing the stabilization and nuclear localization of the important GSK-3β substrate called as β-catenin. Our data shows that Li^+ treatment promotes nuclear localization of β-catenin in NIH/3T3 and A172 cells. β-catenin is an important constituent of the Wnt signaling pathway. The Li^+ induced nuclear localization of β-catenin is an expected result because Li^+ mimcs the action of Wnt ligands (Stambolic et al., 1996; Klein and Melton, 1996; Hedgepeth et al., 1997). Li^+ treatment also regulates the glycogen metabolism pathway. Be^{2+} treatment induced a minimal nuclear translocation of β-catenin in NIH/3T3 and A172 cells (Fig. 17&18). The analysis of the nuclear localization of β-catenin in Be^{2+} or Li^+ treated NIH/3T3 and A172 cells suggests that in comparison to Li^+ induced nuclear localization of β-catenin the effect of Be^{2+} is subtle. Insulin did not elicit any major influence on the nuclear localization of β-catenin and the same has been reported in other studies (Ding et al., 2000).

The inability of Be^{2+} to induce substantial nuclear localization of β-catenin while decreasing the levels of phospho-glycogen synthase points to the fact that Be^{2+} could be a pathway specific GSK-3β inhibitor. Analysis of the effect of Be^{2+} on axin, an important constituent of the β-catenin destruction complex shows that Be^{2+} has little effect on the β-catenin destruction complex, unlike Li^+ (Fig. 21). With little effect on the stability of axin, Be^{2+} will not be able to destabilize β-catenin destruction complex. A stable and active β-catenin destruction complex marks β-catenin for ubiquitin mediated proteosomal degradation thus deterring any substantial nuclear localization of β-catenin. GSK-3β enzyme can be dived into two cellular fractions the “free GSK-3β”, which is part of the insulin signaling pathway and the “bound GSK-3β” involved in the Wnt pathway. Li^+ seems to be behaving as a GSK-3β inhibitor in a more universal fashion, inhibiting both the free GSK-3β and the β-catenin destruction complex bound GSK-3β fractions. The GSK-3β associated with the β-catenin destruction complex is responsible for phosphorylating β-catenin and marking it for ubiquitin mediated proteasomal degradation.

We hypothesized that the lack of nuclear localization of β-catenin in the presence of beryllium is because of its inability to regulate the "bound GSK-3β" which is part of the β-catenin destruction complex. Axin is a member of the β-catenin destruction complex along with APC, PP2A, GSK-3β and CK-1. Phosphorylation of axin by GSK-3β stabilizes it and plays a central role in the regulation of β-catenin. Analysis of the effect of Li^+ on axin stability shows that Li^+-treatment caused de stabilization of axin in A172 and NIH/3T3 cells (Fig 21a&b, panel 1). However Be^{2+} seems to be having no effect on axin stability in A172 and NIH/3T3 cells (Fig 21a, b&c). Lithium is known to de stabilize axin by interfering with GSK-3β mediated phosphorylation of axin (Yamamoto et al., 1999). It can be concluded that Li^+ is able to inhibit the kinase activity of "bound GSK-3β" because of which it cannot phosphorylate axin. However Be^{2+} seems to be unable to interfere with the kinase activity of "bound GSK-3β" thus eliciting no effect on axin's stability. It can be hypothesized that the Be^{2+} induced decrease of pGS is because of the Be^{2+} mediated inhibition of "free GSK-3β". Conversely the inability of Be^{2+} to elicit the nuclear localization of β-catenin could be because of its failure to access or inhibit the "bound GSK-3β" thus leaving its kinase activity intact. The inability of Be^{2+} to regulate proteins like axin and β-cartenin indicates that the Wnt pathway could be insulated from its inihibitory effects.

Various studies have demonstrated the possibility of a cross talk between p53 and β-catenin and that the activation of p53 expression down regulates β-catenin (Damalas et al., 1999; Sadot et al., 2001; Levina et al., 2004). In our study it was observed that Be^{2+} could not induce nuclear localization of β-catenin; we thought it would be interesting to investigate the effect of p53 upregulation on the nuclear localization of β-catenin. Our results demonstrated that in cell lines carrying wt p53 Be^{2+} treatment did not induce any nuclear localization of β-catenin (Fig. 16). Be^{2+} treatment causes up regulation of p53 expression (Lehnert et al., 2001; Coates et al., 2007; Gorjala and Gary, 2010) and it could probably be one of the reasons for the lack of nuclear localization of β-catenin. Down regulation/knock down of p53 resulted in clear nuclear

localization of β-catenin (Fig. 22, 24, 27&28). De regulation of β-catenin plays a central role in development of various cancers. Hence β-catenin is regulated by multiple, overlapping and intricate mechanisms which are still not clearly understood; p53 mediated down regulation of β-catenin is one among them. It has been proposed that p53 communicates with the endogenous β-catenin using a micro RNA known as miRNA-34a (Kim et al., 2011; Siemens et al., 2013). Our efforts to knock down miRNA-34a using specialized RNAi analog to establish the role of miR-34a in p53 mediated blockade of nuclear localization of β-catenin was not successful (data not shown).

Li^+ induced decrease of phospho β-catenin in 293T cells (human embryonic kidney cells) has been reported previously (Sadot et al., 2002). Our studies indicate that in A172 cells (human glioblastoma) Li^+ treatment at 10 or 20 mM concentration leads to an increase in phospho-β-catenin levels. (Fig.29). Be^{2+} treatment on the other hand has no effect on the phospho-β-catenin (Fig. 29). Even though Be^{2+} and Li^+ are both inhibitors of GSK-3β they seem to be playing contrasting roles in relation to the phosphorylation status of β-catenin. Interestingly no change was observed in the total β-catenin levels even when the phosphorylated form of endogenous β-catenin was up regulated. This observation suggests that the change is primarily in the phosphorylation status of β-catenin. Recently it was shown that Wnt stimulation elicited accumulation of phosphorylated form of β-catenin in HEK293T cells (Gerlach et al., 2014). Lithium mimics the action of Wnts (Stambolic et al., 1996; Hedgepeth et al., 1997) and the Wnt signaling induced accumulation of phospho-β-catenin in HEK293T cells gives credibility to the ability of lithium to up regulate phosphorylated form of β-catenin.

It has been suggested that along with GSK-3β mediated phosphorylation there could be some serine/threonine phosphatases which are involved in the regulation of β-catenin. Protein phosphase 2A (PP2A) is an prominent member of the β-catenin destruction complex and plays an important role in the regulation of Wnt/β-catenin signaling pathway (Willert et al., 1999; Seeling

et al., 1999; Li et al., 2001; Yang et al., 2003; Yokoyama and Malbon, 2007). PP2A is made up of three subunits designated as A, B and C which constitutes the holoenzyme. It has been reported that the phosphorylation of PP2A inhibits its phosphatase activity, which in turn leads to an increase in β-catenin phosphorylation (Bos et al., 2006). Interestingly lithium has been shown to inhibit PP2A in certain cell types (Mora et al., 2002). It has been reported that lithium inhibits PP2A by preventing methylation of subunit C and by causing the dissociation of subunit B from the core enzyme (Chen et al., 2006). These studies points to the fact that lithium has the ability to regulate the phosphatase activity of PP2A towards phospho-β-catenin. Another closely related phosphatase called as protein phosphatase 1 (PP1) is an important constituent of the GSK-3β–TIMAP–PP1 feedback loop, wherein it positively regulates GSK-3β via its phosphatase activity (Jope, 2003; Li et al., 2007). The ability of Li^+ to elicit an increase in the Ser-9 phosphorylation of GSK-3β has been partly attributed to the perturbations it can cause in the GSK-3β–TIMAP–PP1 feedback loop (Zhang et al., 2003). It is possible that only Li^+ but not Be^{2+} is able to interfere with the PP2A activity, because of which only Li^+ could be facilitating accumulation of phospho-β-catenin in A172 cells.

However the precise role of PP2A in the β-catenin destruction complex is not clear yet. It has been reported that PP2A might be involved in de phosphorylation of APC (Ikeda et al., 2000). The most important question whether PP2A can dephosphorylate the β-catenin associated with the β-catenin destruction complex remains unanswered. It has been reported that PP2A can dephosphorylate β-catenin in vitro (Su et al., 2008). Su et al also suggests that APC could be protecting the phosphorylated N-terminal region of β-catenin by shielding it from PP2A. The available data implies that the role of PP2A in regulation of β-catenin in the β-catenin destruction complex is not clearly understood.

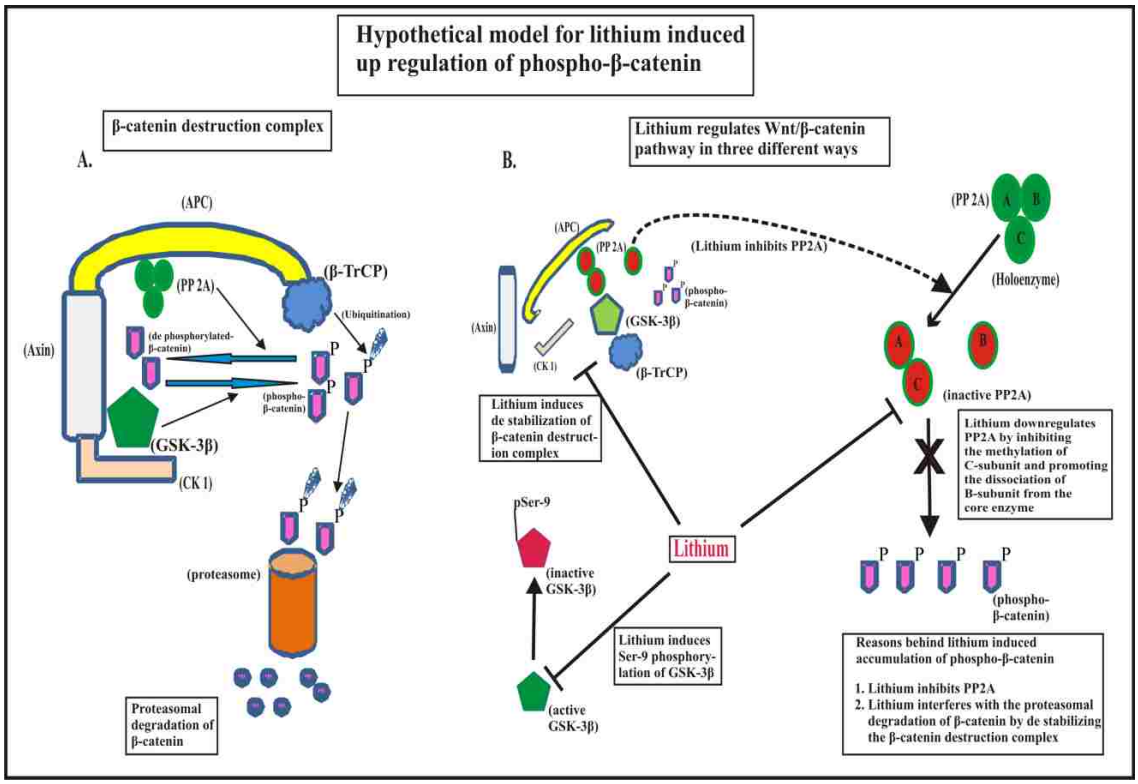
β-TrCP is a F-Box protein which is an important component of the ubiquitin ligase complex (SCF) involved in regulation of β-catenin stability (Winston et al., 1999; Latres et al., 1999) and

this ubiquitin ligase complex is associated with the β-catenin destruction complex (Hart et al., 1999; Liu et al., 1999, Major et al., 2007). The GSK-3β mediated phosphorylation of Ser-33 and Ser-37 present on the N-terminal region of β-catenin in the β-catenin destruction complex serves as the recognition site for β-TrCP (reviewed in Kikuchi et al., 2006). The β-TrCP initiates the binding of E2 ligase followed by poly-ubiquitination of β-catenin which ultimately leads to its proteosomal degradation (reviewed in Kimelman and Xu, 2006). It has been suggested that APC could be playing an important role in the β-TrCP mediated ubiquitination of β-catenin (Sadot et al., 2002; Yang et al., 2006; Su et al., 2008). It has also been suggested that in the absence of APC, phoshorylated form of β-catenin is rapidly de phosphorylated thus diminishing the possibility of poly-ubiquitination (Su et al., 2008). These studies suggest that some members of the β-catenin destruction complex could be helping the β-TrCP mediated ubiquitination of β-catenin. Hence the loss of a stable and functional β-catenin destruction complex could interfere with the β-TrCP mediated ubiquitination thus preventing the proteosomal degradation of phospho-β-catenin.

Lithium is a strong GSK-3β inhibitor and is known to inhibit GSK-3β's ability to phosphorylate its substrates. β-catenin is a well established and highly studied GSK-3β substrate and the "accepted β-catenin dogma" is that the inhibition of GSK-3β should elicit a sharp decrease in phospho-β-catenin levels. Surprisingly our results suggest that the "accepted β-catenin dogma" may not be always true. Lithium seems to be inhibiting the Wnt/β-catenin signaling pathway by three different mechanisms. Lithium induces Ser-9 phosphorylation of GSK-3β (Zhang et al., 2003), it induces destabilization of axin because of which the stability of the β-catenin destruction complex could be affected (Fig. 21). Lithium also inhibits the activity of PP2A (Chen et al., 2006; Mora et al., 2002). The above mentioned reasons could be responsible for the lithium mediated up regulation of phosphorylated form of β-catenin. We are proposing a hypothetical model to explain the "regulation of β-catenin/phospho-β-catenin" by the β-catenin

destruction complex and the role of lithium as a “Wnt mimicking agent” (**schematic diagram 6, - next page**)

Schematic diagram 6: Hypothetical model explaining the increase of phospho-β-catenin in the presence of lithium



The lithium induced increase in the phospho-β-catenin levels of A172 cells was a surprising observation (Fig. 29). Conventional knowledge about lithium's role as GSK-3β inhibitor points to the fact that it should lead to a decrease in the levels of phospho-β-catenin, as explained in Sadot et al. A critical difference between our study and Sadot et al is their use of 293T cells (human embryonic kidney cells containing the SV40 T antigen) and we have used A172 cells (glioblasoma). Another important difference is the p53 status in A172 and 293T cells; T antigen is known disrupt the activity of p53 protein (Ali and DeCaprio, 2001). We speculated that the accumulation of phospho-β-catenin in A172 cells could be because of the presence of wt p53. Hence we used the shRNA A172 p53 KO cells to analyze the effect of lithium on phospho-β-catenin. Our speculation that the presence of wt p53 in A172 cells could be causing accumulation of phospho-β-catenin in lithium treated cells was incorrect. The effect of lithium on phospho-β-catenin did not change depending upon the functional status of p53. These observations are very intriguing because Li^+ induced inhibiton of GSK-3β should cause a decrease in the phospho-β-catenin. As observed p53 status seems to be eliciting no effect on the Li^+ induced accumulation of phospho-β-catenin. There is a remote possibility that the Li^+ induced accumulation of phospho-β-catenin in A172 cells could be an exclusive cell type specific event. However it would be prudent to examine the effect of Li^+ on phospho-β-catenin in other cell types as well.

Several studies have shown that β-catenin plays a critical role in cancerous proliferation of cells (Morin, 1999; Valenta et al., 2012). De regulation of β-catenin can happen due to many reasons and the two most important being: 1. mutations in the β-catenin gene (CTNNb1) which affects the important serine/threonine residues in the N-terminal region of the β-catenin. 2. Any mutation or inhibition of the proteins like APC, axin, GSK-3β or PP2A, which are all constituents of the β-catenin destruction complex. The mutated ser/thr residues on the amino terminal region of β-catenin cannot provide the post translational phosphorylation signal for the ubiquitin mediated proteasomal degradation of β-catenin. The stabilization and nuclear accumulation of β-

catenin leads to activation of β-catenin-target genes some of which are involved in the development of cancer (Thakur and Mishra, 2013). Activation of Wnt signaling and abnormal nuclear localization of β-catenin results in development of tumoural phenotype in mesenchymal stem cells (Herencia et al., 2012), development of colon cancer (Tetsu and McCormick, 1999) and development of human colorectal carcinomas (Mann et al., 1999). Nuclear localization of β-catenin is associated with the local lymph node metastasis or distant metastasis in a variety of cancers like colorectal (Cheng et al., 2011), breast (Robles-Frias et al., 2006), oesophageal (Hou et al., 2011) and cervical (Noordhuis et al., 2011). Nuclear localization of β-catenin is associated with malignant melanomas as well (Rimm et al., 1999) and abnormal repression of GSK-3β can possibly lead to activation of oncogenes**.**

Inhibition of GSK-3 presents a peculiar problem and has its own implications**.** Down regulation of GSK-3β activity could have a positive effect on the glycogen metabolism and tau protein phosphorylation dynamics, which have direct implications in diabetes miletus type 2 and alzheimer's disease respectively. Conversely inhibition of GSK-3β could lead to activation of proto oncogenes due the stabilization and nuclear localization of β-catenin.

A potent GSK-3β inhibitor is expected to bring about the desired effect along with the stabilization and nuclear localization of β-catenin. As demonstrated in our study GSK-3β inhibitors insulin, Li^+ and SB216763 were successful in down regulating the phosphorylation of GS and except for insulin the other two GSK-3β inhibitors (Li^+ and SB216763) caused nuclear localization of β-catenin as well. Li^+ is already in use for anti-bipolar therapy. The IC_{50} value of Li^+ is ~ 2mM but the therapeutic concentration is ~1 mM (Malhi et al., 2013). The use of Li^+ at a concentration lower than its IC_{50} for therapeutic purpose is to avoid complete or unrestricted inactivation of GSK-3β. Our study indicates that Be^{2+} can act as a potent GSK-3β inhibitor at intra cellular level. The inhibitory effect of Be^{2+} seems to be biased more towards the glycogen synthesis pathway, while keeping the Wnt signaling fairly insulated from its inhibitory effects.

Insulin is a GSK-3β inhibitor, but the GSK-3β fraction which is part of the β-catenin destruction complex seems to be "insulated" from the inhibitory action of insulin (Ding et al., 2003). However the possibility of a cross talk between insulin and Wnt signaling pathway does exists and it has been reported that insulin can stimulate β-catenin pathway (Desbois-Mouthon et al., 2001). Hence it is necessary to use the word "insulated" in relation to GSK-3β inhibition with caution.

This preliminary study highlights the unique properties of Be^{2+} in terms of its differential regulatory effect on the pathways in which GSK-3β plays a central role. This unique property of Be^{2+} could conceivably be exploited for therapeutic purpose. Before commenting on the therapeutic potential of Be^{2+} it is imperative to ascertain the mechanism behind the Be^{2+} induced inhibition of GSK-3β. How is Be^{2+} modulating the inhibition of GSK-3β by "direct mechanism" and what is the effect of Be^{2+} on the "scaffolding proteins" that are part of the β-catenin destruction complex are some of the un answered questions.

CHAPTER 6

Beryllium could be involved in the regulation of BTG2, DDB2 and FAM III B gene products

Introduction:

Our lab demonstrated that beryllium can induce an increase in the expression of important cell cycle regulators p53 and p21 (Coates et al., 2007; Gorjala and Gary, 2010, Mudireddy et al., 2014). There is very limited information available about the effect of beryllium on mammalian cells at molecular level. There could be many other important genes/gene products that might be regulated by Be^{2+}. Microarray is a powerful technique that can help in analyzing the expression of genes under different conditions. From the microarray data available in our lab, it was observed that apart from p21 there are other genes, which respond to beryllium treatment. BTG2, DDB2 and FAM III B genes were selected for this study and the expression of these genes after Be^{2+} treatment was analyzed using RT PCR.

Materials and methods

Cell culture

A172 cells were cultured in RPMI 1640 HEPES (cat#23400-021, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic-mycotic.

Beryllium treatment

$BeSO_4.4H_2O$ (lot&filling code: 413015/1 22001, Fluka) was used to prepare a stock solution. Beryllium stock solution was diluted to obtain required concentration.

Cells used in this study were obtained after trypsinizing a batch of cell culture dishes. A common pool of cells was generated which was then used for the experiment. The cells designated as control were cultured in normal RPMI 1640 or 10 μM $BeSO_4$ for 24 hr.

Real time PCR

After the treatment of A172 with the inhibitors or ionizing radiation, total mRNA was extracted using Rneasy Mini Kit and QIA-shredder (Qiagen cat #74104 and 79654). The RNA concentration was measured using nano drop UV-vis spectrophotometer. Total mRNA was reverse transcribed to generate copies of cDNA. Real time PCR was performed on same quantity of cDNA sample using QuantiTect SYBR green PCR kit (Qiagen cat #205311) and QuantiTect SYBR green PCR kit (Qiagen cat # 204143). The following primer sets were used in the study QT00095431, QT00079247, QT00062090, QT00240247 for human GAPDH, human p21 (CDKN1A) and human BTG2 respectively (QuantiTect Primer assay). .

The thermal cycling program was set in the following way for amplification and detection –

Step 1: Initial denaturation step at 95℃ for 15 minutes

Step 2: Annealing at 55℃ for duration of 30 seconds.

Step 3: The primer extension step was set at 72℃ for 30 seconds

The step 1, 2 and 3 can be considered as a thermal cycle and the BioRad thermal cycler was programmed to repeat this cycle 40 times.

Final step: The thermal cycle program was terminated after performing a final denaturation step at 95℃ for 1 minute followed by a final extension performed at 72℃ for 1 minute

For the melt curve analysis the starting point was set at 55℃ for 10 seconds with a step by increase of 0.5℃ after cycle 2 (of the total 40 cycles).

For data analysis – RT PCR data was normalized against actin. The upregulation or down regulation of the gene expression under the effect of inhibitors or ionizing radiation was calculated as % of control.

IR treatment

For ionizing radiation, the Cabinet X-Ray system, model Rx-650, by Faxitron X-ray corp was used. Accordingly A172 cells were exposed to 5 Gy or 10 Gy ionizing radiation.

Statistical analysis:

GraphPad Prism v6.0c (Mac OS) was used for analyzing the data. One-way ANOVA with Tukey's multiple comparisons test was used to obtain *P* values comparing each group with all the other groups ($P < 0.05$ considered as significant). The log transformed values were used for the analysis.

Results

Be^{2+} at concentrations, as low as 10 µM caused a marked increase in the mRNA levels of the important cell cycle regulator p21 in A172 cells (Fig. 33). Li^{+} treatment too caused an increase in the mRNA levels of p21. However exposing the cells to ionizing radiation (X-rays) did not elicit any increase in the mRNA levels of p21.

Be^{2+} caused a significant decrease in the mRNA levels of FAM III B in A172 cells (Fig. 34). Li^{+} and ionizing radiation (X-ray) treatment as well caused a decrease in the mRNA levels of FAM II B. However the function of FAM III B gene is not known.

DDB2 gene encodes the DNA damage binding protein 2 and it is is involved in DNA damage repair. (Keeney et al., 1993; Wakasugi et al., 2002; Liu et al., 2000). Be^{2+} has no significant effect

on the mRNA levels of DDB2 in A172 cells. Li^+ treatment and exposure of A172 cells to X-ray did not have any significant effect on the DDB2 mRNA levels (Fig. 35).

B-cell translocation gene 2 (BTG2) is a member of the BTG gene family and encodes the BTG2 protein. BTG2 is a p53 dependent protein and is an important component of the cellular response to DNA damage (Rouault et al., 1996; Puisieux and Magaud, 1999; Winkler, 2010). In the presence of Be^{2+} there is little increase in the mRNA levels of BTG2, which is comparable to the increase/change induced by ionizing radtaion (X-ray) treatment (Fig. 36). Li^+ treatment caused significant increase in the mRNA levels of BTG2 gene in A172 cells.

The is no significant change in the actin mRNA levels in the presence of Be^{2+} or after treatment with ionizing radiation (Fig. 37). Li^+ treatment seems to be down regulating the actin mRNA levels to an extent.

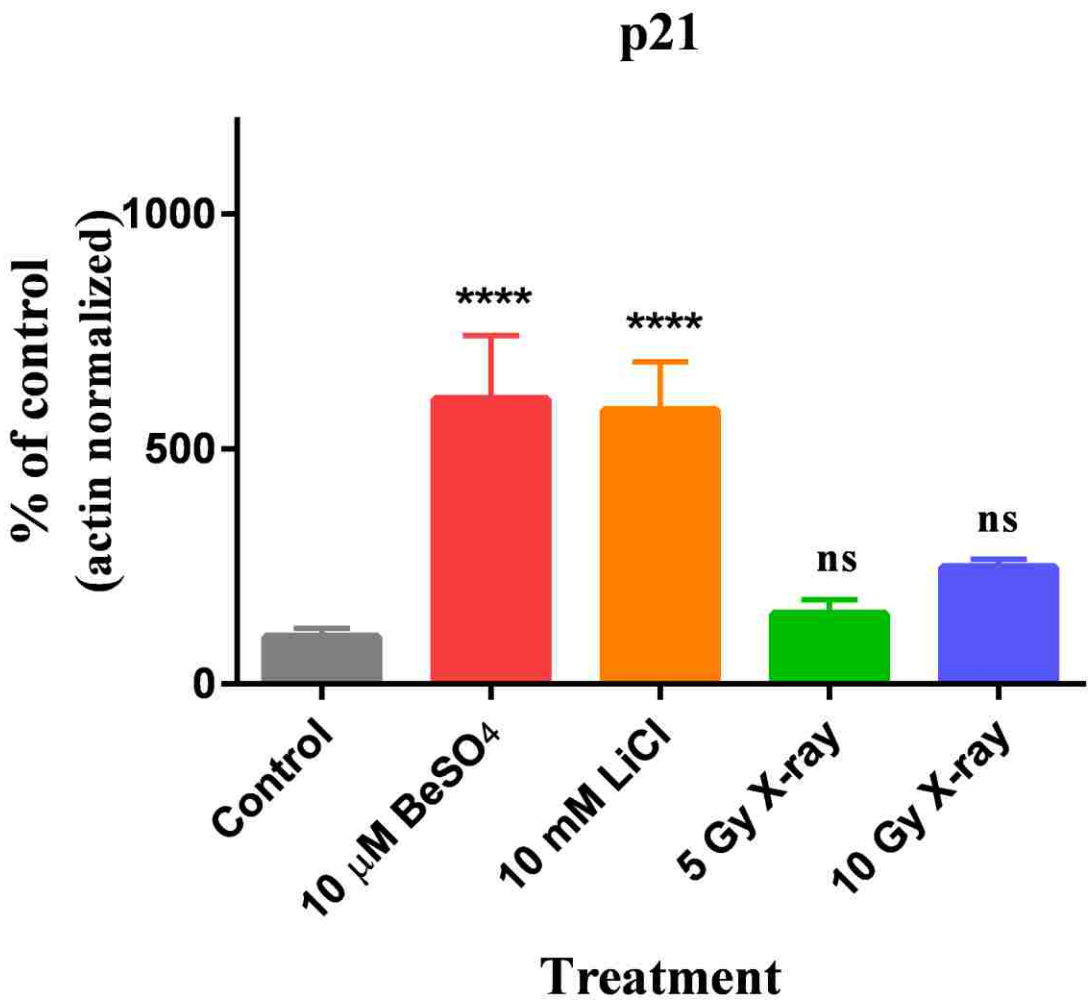


Fig. 33 Beryllium at 10 µM concentration caused an increase in p21. A172 cells were treated with 0, 10 µM $BeSO_4$, 10 mM LiCl for 24 hr or 5 Gy X-ray or 10 Gy X-ray. A significant increase in the p21 mRNA is observed in the 10 µM $BeSO_4$ samples. Statistically significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistically non significant values are mentioned as *ns*.

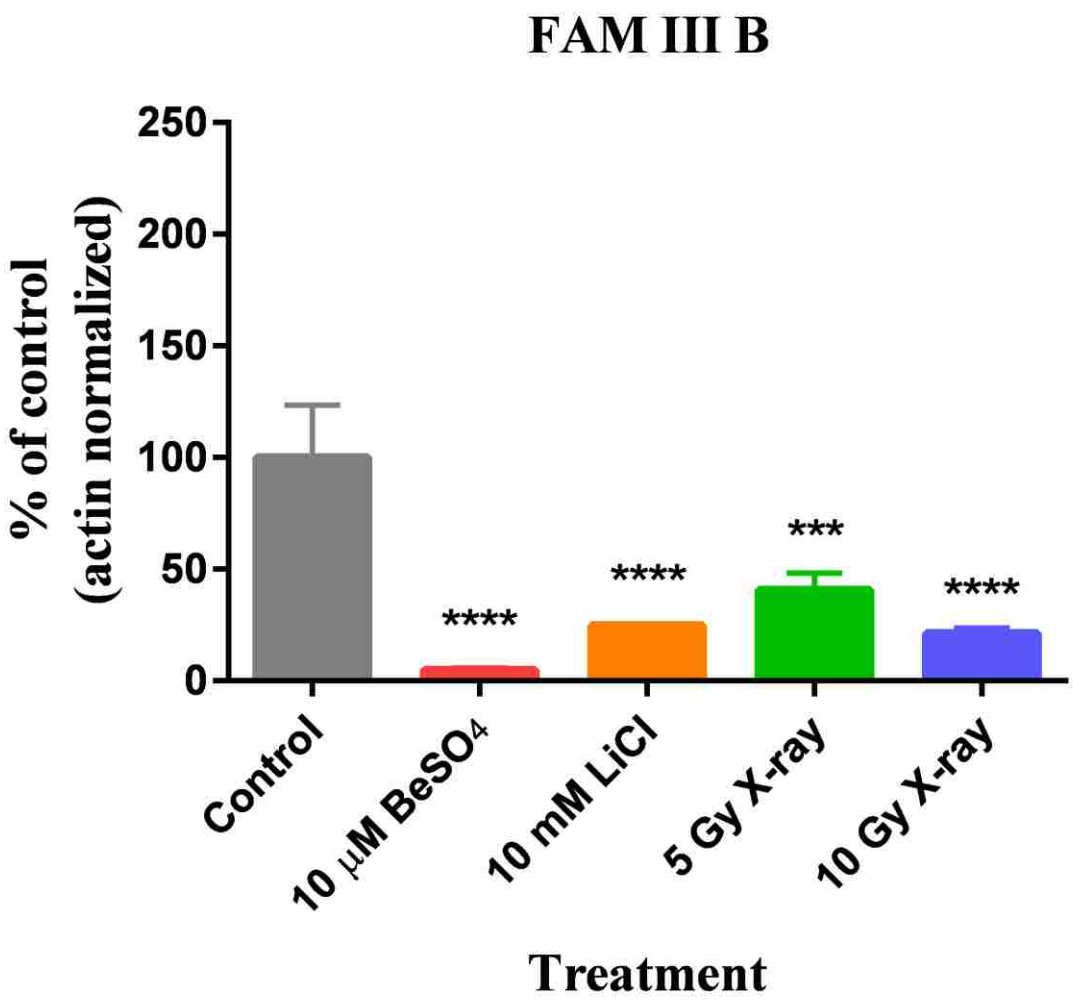


Fig. 34 Beryllium at 10 µM concentration caused a significant decrease in FAM III B mRNA. A172 cells were treated with 0, 10 µM $BeSO_4$, 10 mM LiCl for 24 hr or 5 Gy X-ray or 10 Gy X-ray. Data presented as mean +/- standard deviation. Statistically significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

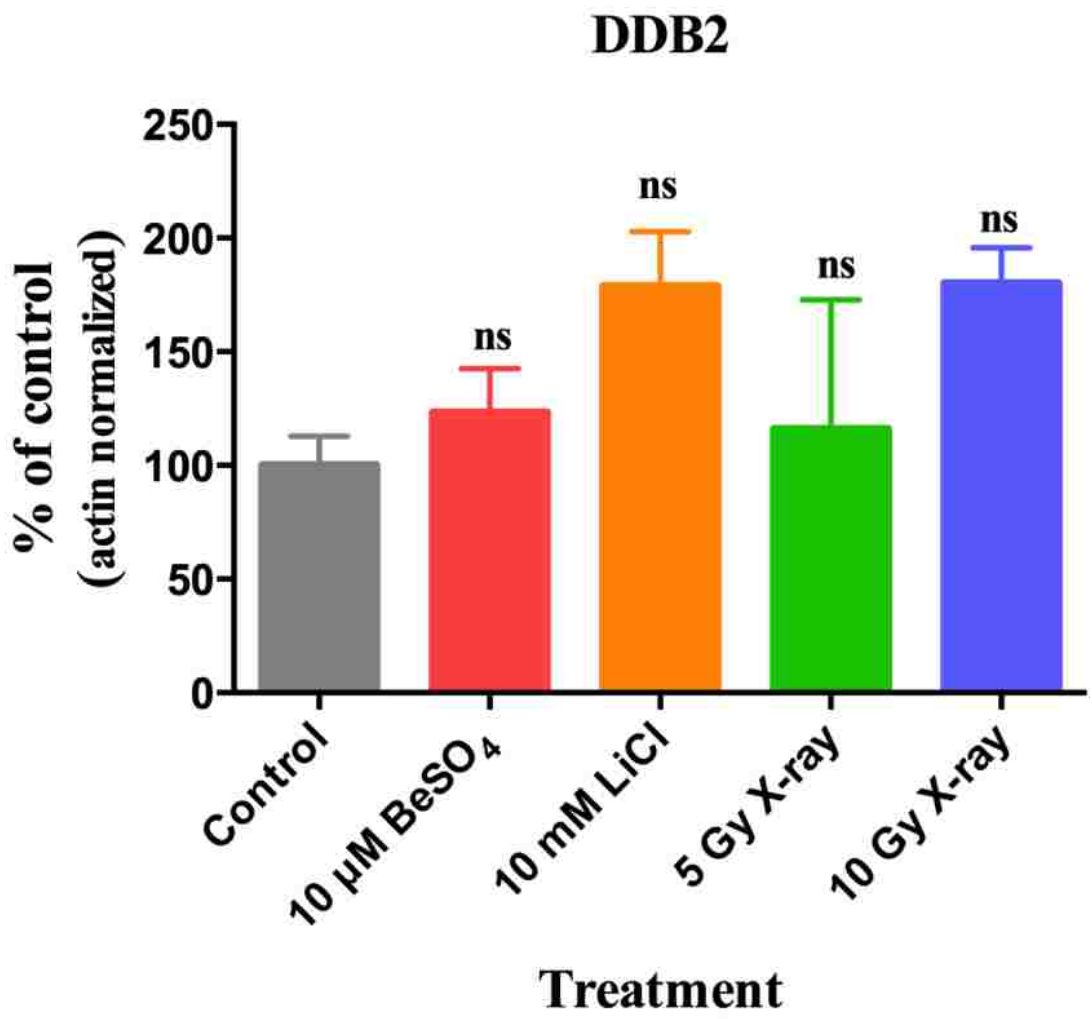


Fig. 35 Beryllium at 10 µM concentration had no significant effect on the DDB2 mRNA. A172 cells were treated with 0, 10 µM $BeSO_4$, 10 mM LiCl for 24 hr or 5 Gy X-ray or 10 Gy X-ray. Data presented as mean +/- standard deviation. Statistically significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistically non significant values are mentioned as *ns*.

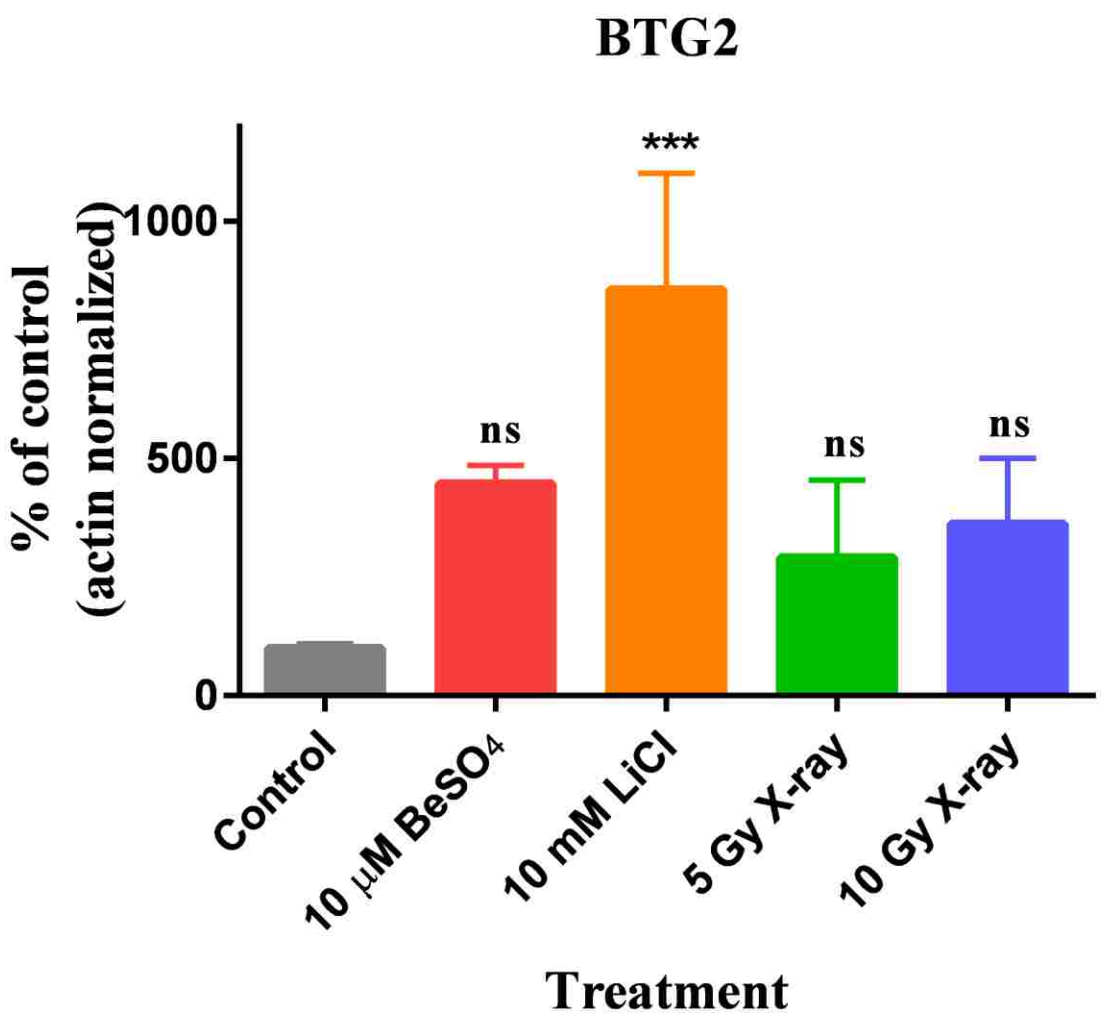


Fig. 36 Beryllium at 10 µM concentration had no significant effect on the BTG2 mRNA. A172 cells were treated with 0, 10 µM $BeSO_4$, 10 mM LiCl for 24 hr or 5 Gy X-ray or 10 Gy X-ray. Data presented as mean +/- standard deviation. Statistically significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistically non significant values are mentioned as *ns*.

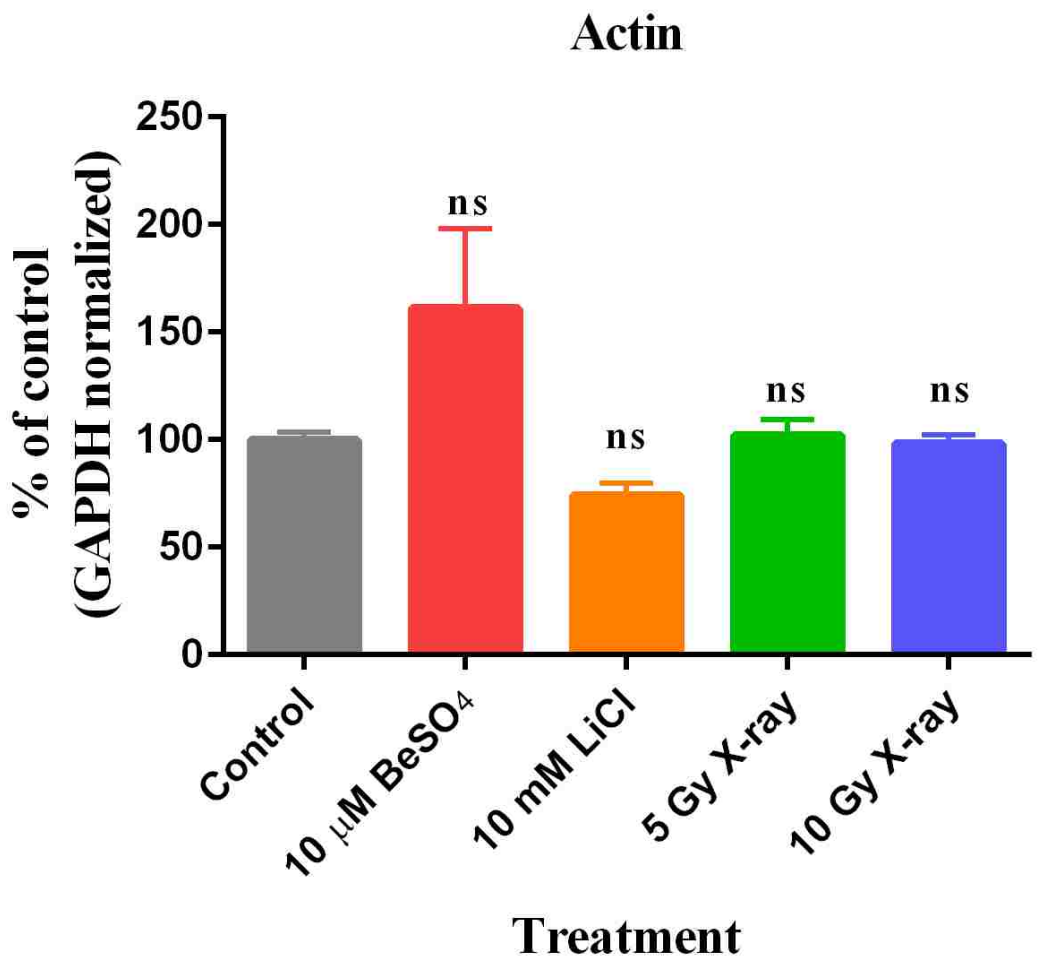


Fig. 37 Beryllium at 10 µM concentration had no significant effect on the actin mRNA levels. A172 cells were treated with 0, 10 µM $BeSO_4$, 10 mM LiCl for 24 hr or 5 Gy X-ray or 10 Gy X-ray. Data presented as mean +/- standard deviation. Statistically significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistically non significant values are mentioned as *ns*.

Discussion

Limited information is available about the effects of beryllium at molecular level. The effect of beryllium on the cell cycle regulators like p53 and p21 has been documented well in our lab. Here we tried to investigate the effect of Be^{2+} on other genes/gene products.

Be^{2+} affects a sharp decrease in the mRNA levels of the FAM III B gene. No information is available about the function of FAM III B gene. In our lab it was observed that beryllium treatment either induces an increase in expression of target genes or has no effect. The significant decrease in the expression of FAM III B gene is an interesting observation, which has to be investigated further.

One of the physiological affects associated with Be^{2+} treatment is the up regulation of cell cycle regulators. DDB2 is a protein associated with DNA damage and the mRNA levels are increasing in the presence of Li^{+} only but not Be^{2+} or ionizing radiation. It indicates that Be^{2+} may not be able to induce any DNA damage in A172 cells. BTG2 protein is associated with cell cycle regulation. Beryllium induces cellular senescence (Coates et al., 2007; Gorjala and Gary, 2010) and the up regulation of BTG2 could be one of the reasons behind Be^{2+} induced senescence.

It is important to analyze the effect of Be^{2+} on the above mentioned genes at the transcript level. It will be worthwhile to investigate whether Be^{2+} can induce any post translational modification or if it has any effect on the stability of the above mentioned gene products.

CHAPTER 7

RESEARCH SUMMARY AND FUTURE DIRECTIONS

Summary

1. The DRC survey shows that not all cell lines are tolerant to beryllium. The breast cancer cell lines MCF-7 and T-47D were found to be tolerant to high concentrations of $BeSO_4$. It was also observed that neuronal cell lines are responsive to optimum concentrations of $BeSO_4$.
2. Beryllium inhibits the in vitro kinase activity of pure recombinant GSK-3β much more potently compared to lithium - another potent inhibitor of GSK-3β. These results indicate a direct interaction between Be^{2+} and GSK-3β.
3. Beryllium is less toxic to cells at physiologically relevant concentrations compared to lithium.
4. The effect of beryllium on the Ser-9 phosphorylation of GSK-3β seems to be cell type specific. Beryllium did not induce an increase in the pSer9-GSK-3β in A172, HFL and U87MG cells. However an increase in the pSer9-GSK-3β was observed in NIH/3T3 and Caki-1 cells.
5. For the very first time our results demonstrate the effect of beryllium on the proteins that are downstream of GSK-3β. A beryllium induced decrease in the phosphorylation status of glycogen synthase (GS) was observed in NIH/3T3 cells. The inhibitory effect of beryllium on the kinase activity of GSK-3β is confirmed by the decrease in the phospho-GS levels.
6. A very interesting aspect of this study has been the effect of beryllium on β-catenin, another important target protein of GSK-3β. Beryllium seems to be differentially regulating glycogen synthase and β-catenin. Lack of nuclear localization of β-catenin in NIH/3T3 and A172 cells in the presence Be^{2+} indicates that the Wnt/β-catenin pathway could be insulated from its effects.

7. Beryllium seems to be having no effect on the stability of axin, an important member of the β-catenin destruction complex. The inability of beryllium to de-stabilize the β-catenin destruction complex could be the possible reason behind its differential regulation of glycogen synthase and β-catenin.
8. We have observed in our study that the presence of active and functional p53 plays an important role in the regulation of β-catenin. Loss of functional p53 caused an increase in the nuclear localization of β-catenin in A172, A172 E6 and U87MGE6 cells.
9. Beryllium was not able to modulate any change in the phosphorylation status of β-catenin. We observed that lithium seems to be inducing an increase in the phospho-β-catenin levels and beryllium cannot. This was a very surprising observation since lithium is a strong GSK-3β inhibitor and is expected to induce decrease in the phosphorylated form of β-catenin. These results also indicate that beryllium could possibly be having no effect on the Wnt/β-catenin pathway. We have proposed a hypothetical model to explain the lithium induced up regulation of β-catenin.

FUTURE DIRECTIONS

1. In order to analyze the effect of Be^{2+} on other cellular kinases, we investigated its effects on the kinase activity of pure recombinant protein kinase A (PKA) enzyme. Our studies (documented in CHAPTER 4) shows that Be^{2+} is down regulating the activity of pure recombinant PKA (Fig. 3a). The specificity of beryllium against the activity of other closely associated kinases like Akt/PKB has to be investigated thoroughly. Akt (also called protein kinase B) phosphorylates GSK-3β and is involved in the insulin signaling pathway (Sutherland et al., 1993; Shaw, et al., 1997; Cross et al., 1994, 1995; Stambolic and Woodgett, 1994). Apart from Akt/PKB another good candidate to analyze the effect of beryllium on other kinases would be the cyclin dependent kinases (CDK). The role of CDKs along with GSK-3β especially CDK-5 has been established in the tau hyperphosphoryaltion

(Plattner et al., 2006; Garcia-Perez et al., 1998). It will be interesting to investigate the inhibitory effect of beryllium on kinases closely associated with GSK-3β, in order to comment on the specificity of Be^{2+}.

2. The inhibitory Ser-9 phosphorylation plays a significant role in regulation of GSK-3β. We observed that beryllium modulates an increase in the Ser-9 phosphorylation of GSK-3β depending on the cell type. It would be logical to investigate whether beryllium induces any other post translational modification of GSK-3β. Phosphorylation of Tyr-216 along with the Ser-9 seems to play an important role in regulating GSK-3β (Diehl et al., 1998; Meijer et al., 2004; Bijur and Jope, 2003; Park et al., 2013). It could be possible that the effect of beryllium on the Tyr-216 if any might be consistent across all cell types. Hence it would be worthwhile to investigate the effect of beryllium on Tyr-216 phoshorylation of GSK-3β.
3. Li^{+} mimics insulin and has a positive effect on glycogen metabolism (Cheng et al., 1983; Bosch et al., 1986; Choi et al., 2000). Beryllium too inhibits GSK-3β and induces a decrease in the inactive phosphorylated form of GS (Fig. 16). However whether the beryllium induced decrease in the phospho-GS form translates into any positive effect on glycogen metabolism or glucose transport has to be investigated further. We have analyzed the effect of Be^{2+} on the phospho-GS levels in murine embryo fibroblasts only and it would be worthwhile to extend these studies in cell lines representing liver and muscle lineages. Since muscle and liver are major sites of glycogen metabolism.
4. A major concern that arises due to lithium induced inactivation of GSK-3β is the nuclear accumulation of β-catenin. The adverse effect of accumulated nuclear β-catenin is the activation of the β-catenin/TCF/LEF target genes, some of which are proto-oncogenes (Dale, 1998; Brantjes et al., 2002). We observed that beryllium seems to be inducing minimal nuclear localization of β-catenin in NIH/3T3 and A172 cells (Fig. 17). However the effect of beryllium on the TCF/LEF target genes which are further down stream of β-catenin, needs to be investigated. The effect of beryllium on the the β-catenin/TCF/LEF targets has to be

investigated to re assure the fact that the beryllium induced inhibition of GSK-3β will not lead to activation of oncogenes. Some of the important β-catenin/TCF/LEF targets that can be investigated are CDK-1, matrix metalloproteinase-7, *c-jun* and *fra-1* (Shtutman et al., 1999; Brabletz et al., 1999; Mann et al., 1999).

5. GSK-3β is an important member of the β-catenin destruction complex along with APC (Adenomatous Polyposis Coli), axin, casein kinase 1 (CK1), protein phosphatase 2A (PP2A) and E3-ubiquitin ligase β-TrCP (Zeng et al., 1997; Behrens et al., 1998; Hart et al., 1998, Ikeda et al., 1998; Itoh et al., 1998; Salic et al., 2000; Kikuchi, 1999). We have analyzed the effect of beryllium on the stability of axin and it was observed that unlike lithium, beryllium was not able to destabilize axin (Fig. 21). APC is another important member of the β-catenin destruction complex and a bonafide substrate of GSK-3β (Ikeda et al., 2000; Ferrarese et al., 2007). It will be interesting to investigate the role of beryllium in regulating the phosphorylation/stability of APC and the overall effect it has on the stability of the β-catenin destruction complex. A comprehensive understanding of the effect of beryllium on the important members of the β-catenin destruction complex could provide answers behind the inability of beryllium to induce clear nuclear localization of β-catenin.

CHAPTER 8

Alternate hypothesis – possible mechanism for Be^{2+} transport into the cells

Bivalent metal cations such as Mg^{2+} and Ca^{2+} play important roles as co factors of proteins/enzymes along with other cations like Zn^{2+}. The concentrations of these cations have to be very tightly regulated because the intra and extra cellular concentrations of these ions are critical for cellular signaling. There are specific mechanisms by which these ions are transported in and out of the cells. There are other metal ions like iron that are also important for cellular functions but we will limit our discussion to the Mg^{2+} and Ca^{2+}. Beryllium is a group IIa metal cation with an ionic radii of 34 pm and is a potential GSK-3β inhibitor at intracellular level (unpublished data). For a metal ion or an inhibitor to function as a biologically active agent it is necessary that the inhibitor has to be internalized by the cell so that it can interact with the target protein. In our lab it was observed that A172 (human glioblastoma) and RKO (human colon carcinoma) cells were able to successfully internalize Be^{2+} from the surrounding media (Gorjala and Gary, 2010). The important question is what is the mechanism by which beryllium is transported into the cell.

It could be possible that the cellular machinery associated with Ca^{2+} or Mg^{2+} transport might be playing an important role in the intracellular transport of beryllium as well.

Calcium ion:

Ca^{2+} is a divalent metal cation that is involved in the regulation of different cellular functions and is also an important intracellular messenger (Carfoli, 2003; Petersen et al., 2005; Berridge, 2005). The intra cellular concentration of Ca^{2+} is approximately 100 nM whereas the extracellular concentration is around 2 mM (Clapham, 2007). The intracellular and extracellular concentrations

of Ca^{2+} are very tightly regulated and the intra cellular Ca^{2+} ions are stored in the endoplasmic reticulum, mitochondria and golgi complex (explained in Bootman, 2012). The Ca^{2+} homeostasis is maintained with the help of multiple transporters such as voltage gated channels, Na^{+}/Ca^{2+} exchanger (NCX), plasma membrane Ca^{2+} ATPase (PMCA), Na^{+}/Ca^{2+} - K+ exchanger (NCKX) and sarcoendoplasmic reticular Ca^{2+} ATPase (SERCA) (Clapham, 2007; Bootman, 2012).

The EF hand domain is the most common motif associated with the Ca^{2+} binding proteins (Nakayama and Kretsinger, 1994). Calmodulin is one of the most well studied Ca^{2+} binding proteins consisting of the EF hand motif (Stevens, 1983; Chin and means, 2000). Various proteins have calmodulin binding sites where it acts as an adaptor recruiting Ca^{2+} to the target proteins that lack the Ca^{2+} binding ability (Chin and Means, 2000).

Calmodulin is classified as a Ca^{2+} binding protein and it can be expected that its binding is fairly specific towards Ca^{2+}. It has been observed that calmodulin has auxillary binding sites for other divalent cations like Zn^{2+} and Mn^{2+} (Milos et al., 1989). However in an independent study by Ozawa et al it was reported that calmodulin can bind to cations with ionic radii larger than Ca^{2+}, such as Ba^{2+} and Pb^{2+} but not smaller ions like Mg^{2+}, Zn^{2+} or Cu^{2+} (Ozawa et al., 1999). It might be possible that calmodulin binds to certain divalent cations like Be^{2+} apart from Ca^{2+} and that Be^{2+} might interfere with the functions of calmodulin.

Apart from calmodulin it could be possible that certain Ca^{2+} ion exchanger complex might be playing a potential role in the transport of Be^{2+} into the cells. It was observed that a Na^{+}/Ca^{2+} -Li exchanger (NCLX) called as FLJ22233 can transport Li^{+} into the cells and affect Ca^{2+} efflux (Patly et al., 2004). Even though Li^{+} is a monovalent cation it is being transported by a transporter associated with Ca^{2+}. It can be speculated that as in the case of Li^{+}, 'ion exchangers' associated with Ca^{2+} transport could be involved in transport of Be^{2+}.

Additionally it can be conjectured that there might be an unidentified 'ion exchanger' that could be involved in the transport of Be^{2+}.

Magnesium ion:

Magnesium is a divalent cation with ionic radii of 133 pm. Mg^{2+} is an important cofactor for various metabolically important enzymes and GSK-3β is one among them. The intracellular concentration of Mg^{2+} ions vary from 17 to 20 mM (Romani, 2007). Mg^{2+} plays an important role in the regulation of proteins and in maintaining the integrity of nucleic acids and phospholipids (Romani and Maguire, 2002).

There are different Mg^{2+} ion transporters/channels associated with prokaryotes and prominent among them are the CorA super family consisting of CorA, ALR1/ALR2, Mrs2/AtMrs2, Lpe10 (Hmiel et al., 1986, 1989; Moomaw and Maguire, 2008). Another Mg^{2+} transporter associated with prokaryotes is the Mgt family consisting of MgtA and MgtB (Smith et al., 1995, 1998; Snavely et al., 1991; Moomaw and Maguire, 2008).

Some of the Mg^{2+} transporters of eukaryotic origin belong to the TRPM family consisting of TRPM6, TRPM7 (LTRPC7, TRP-PLIK) (Alexander et al., 2008; Chubanov et al., 2005; Schlingmann et al., 2002; Schmitz et al., 2002)

The CorA transporter is known to transport Co^{2+} and Ni^{2+} apart from Mg^{2+} ions (Gibson et al., 1991). However the Mg^{2+} ion transporters are quite selective towards Mg^{2+} ions, which are mostly hexacoordinated (Maguire and Cowan, 2002) and Be^{2+} ions on the other hand are tetra coordinated with solvent ligands (Pittet et al., 1990). It could be possible that the transporters associated with Mg^{2+} might selectively exclude the tertacoordinated Be^{2+} ions.

However the argument that Ca^{2+} and Mg^{2+} transporters could be involved in the transport of Be^{2+} is speculative and has to be supported with experimental evidence.

Table 8. Ionic radii of metal ions

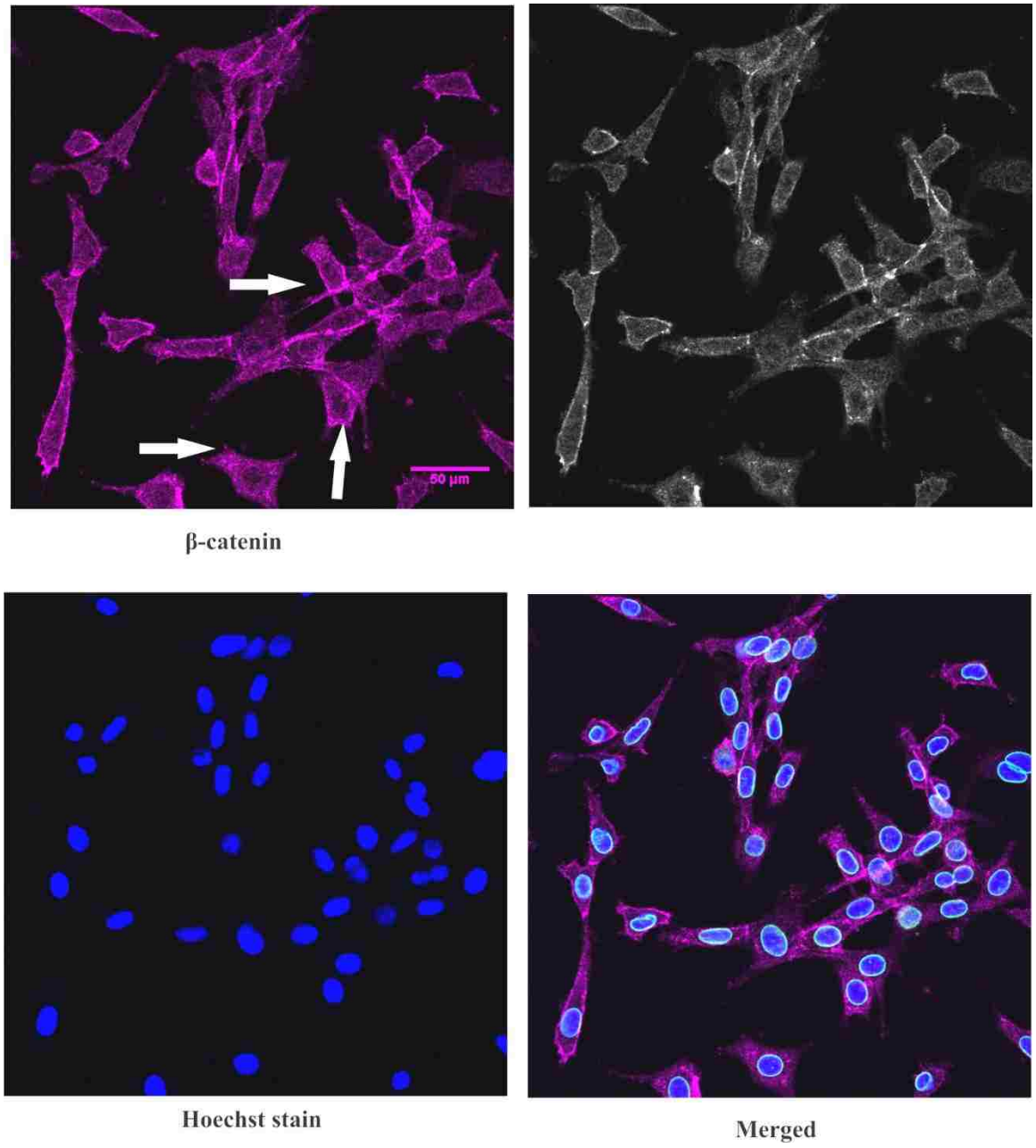
Atomic number	Name	Atomic radii (pm)	Ionic radii (pm)
3	Lithium	152	78
4	Beryllium	113	34
11	Sodium	186	98
12	Magnessium	160	79
19	Potassium	227	133
20	Calcium	197	106

(Data taken from John Emsley, *The Elements*, 3rd edition. Oxford: Clarendon Press, 1998)

Supplementary Figure I

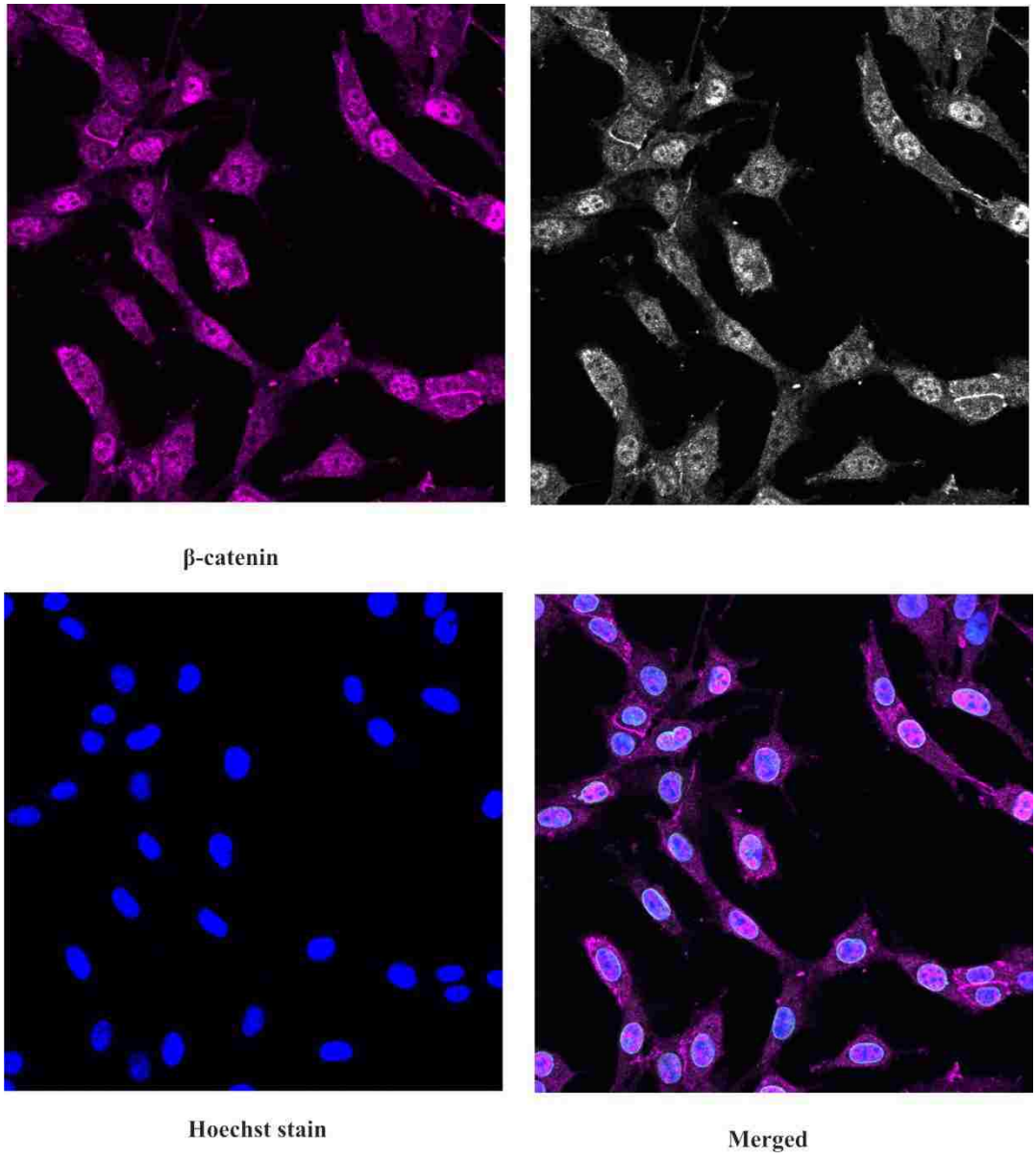
Individual immunoflorescence images corresponding to the Fig. 17a from Chapter 5.

NIH/3T3 control/untreated cells (arrows indicate nuclear β-catenin)



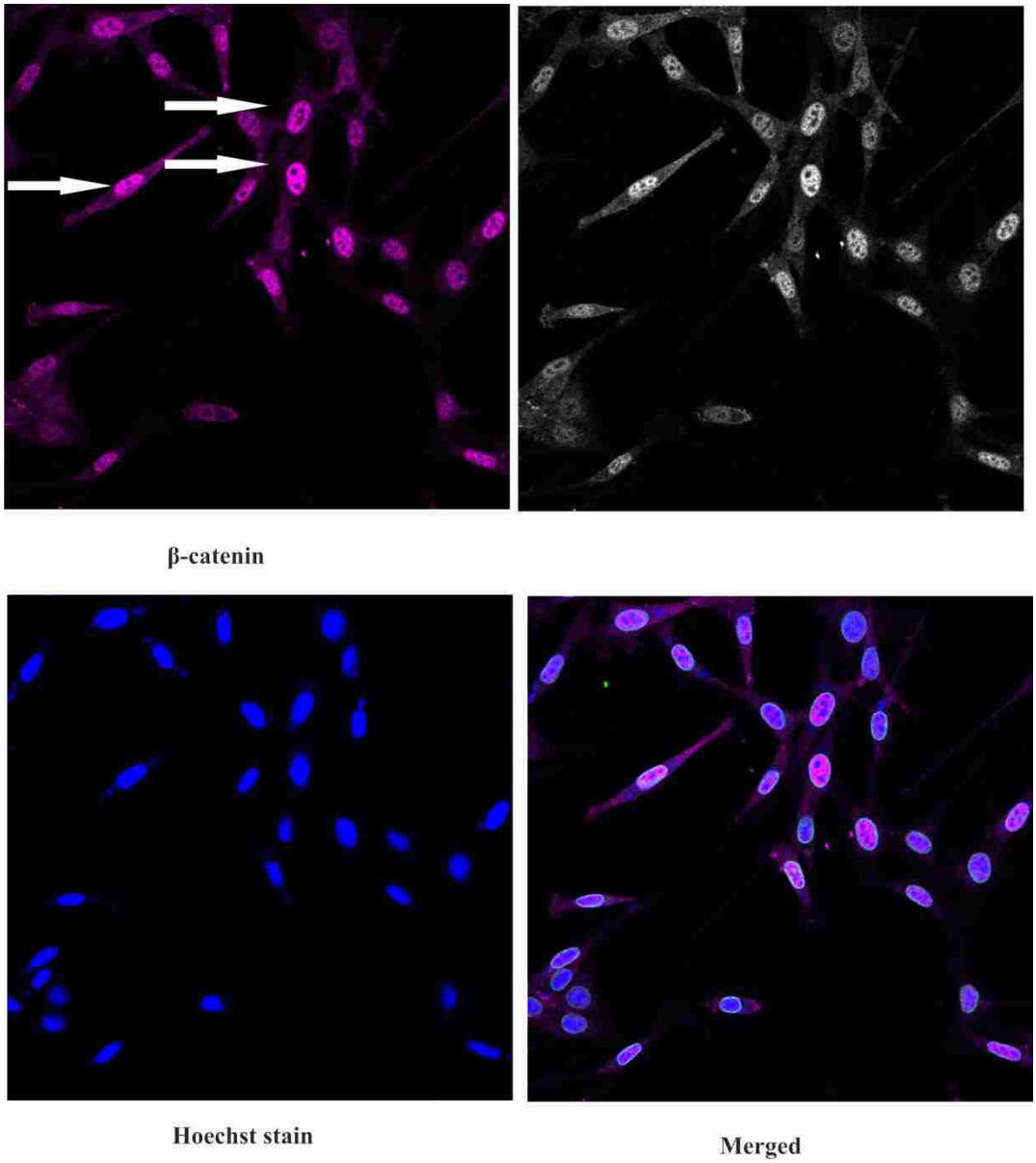
Supplementary figure Ia: Untreated NIH/3T3 cells

NIH/3T3 cells treated with 20 mM LiCl (profound nuclear localization of β-catenin)



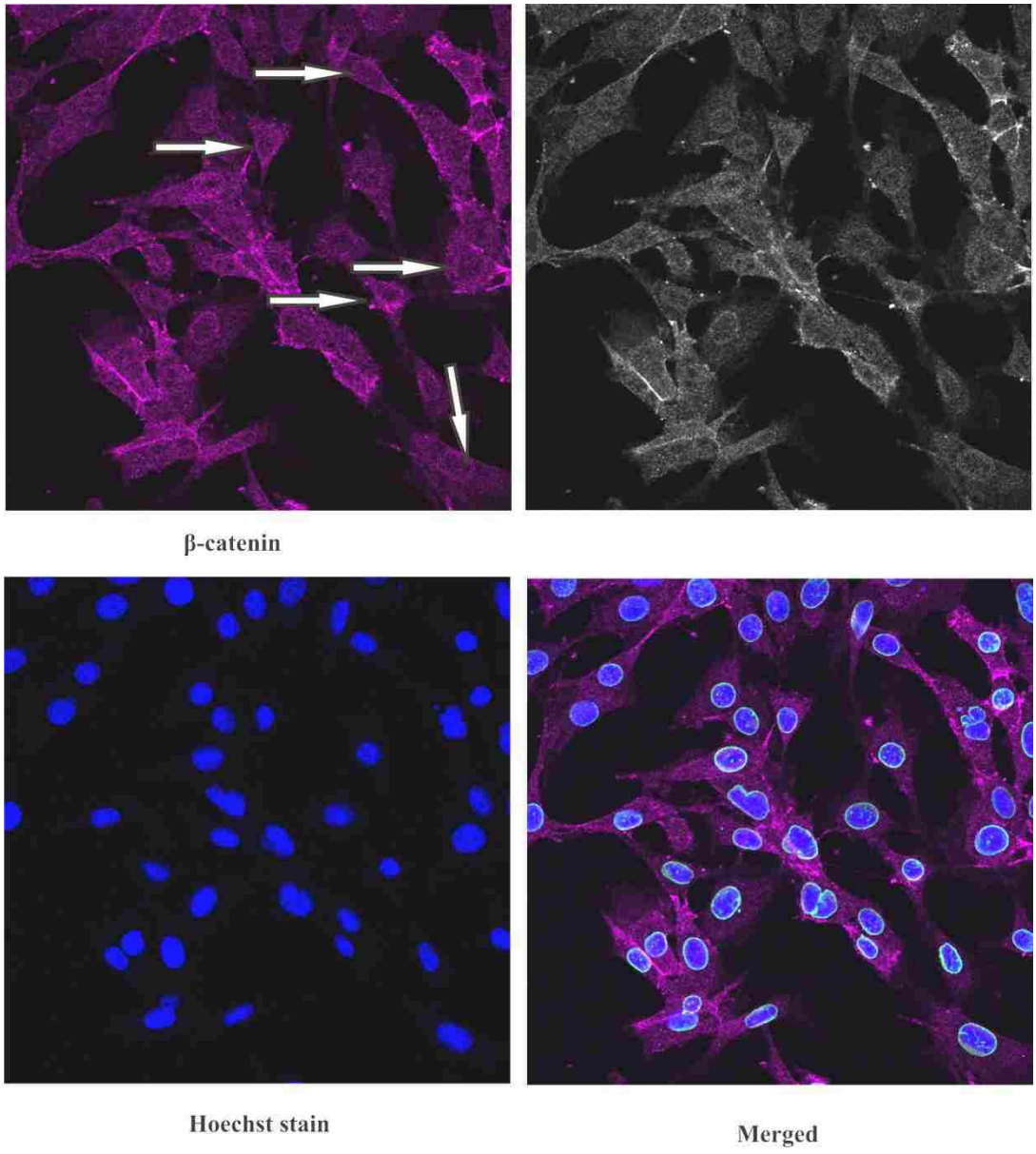
Supplementary figure Ib: NIH/3T3 cells treated with 20 mM LiCl for 24 hr.

NIH/3T3 cells treated with 20 µM SB216763 (profound nuclear localization of β-catenin)



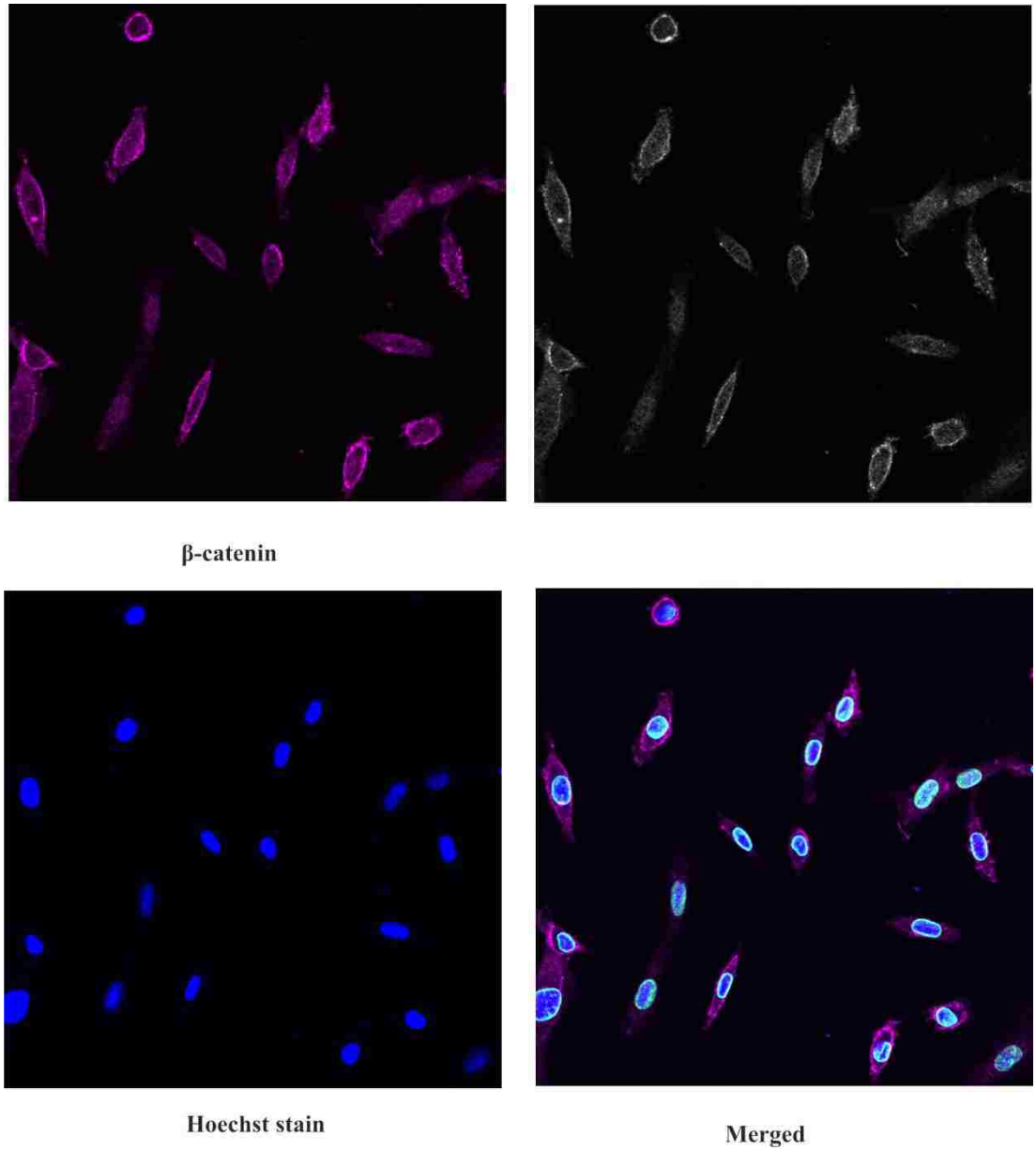
Supplementary figure Ic: NIH/3T3 cells treated with 20 µM SB216763 for 24 hr

NIH/3T3 cells treated with 100 µM $BeSO_4$ (minimal nuclear localization of β-catenin)



Supplementary figure Id: NIH/3T3 cells treated with 100 µM $BeSO_4$ for 24 hr

NIH/3T3 cells induced with 100 nM Insulin

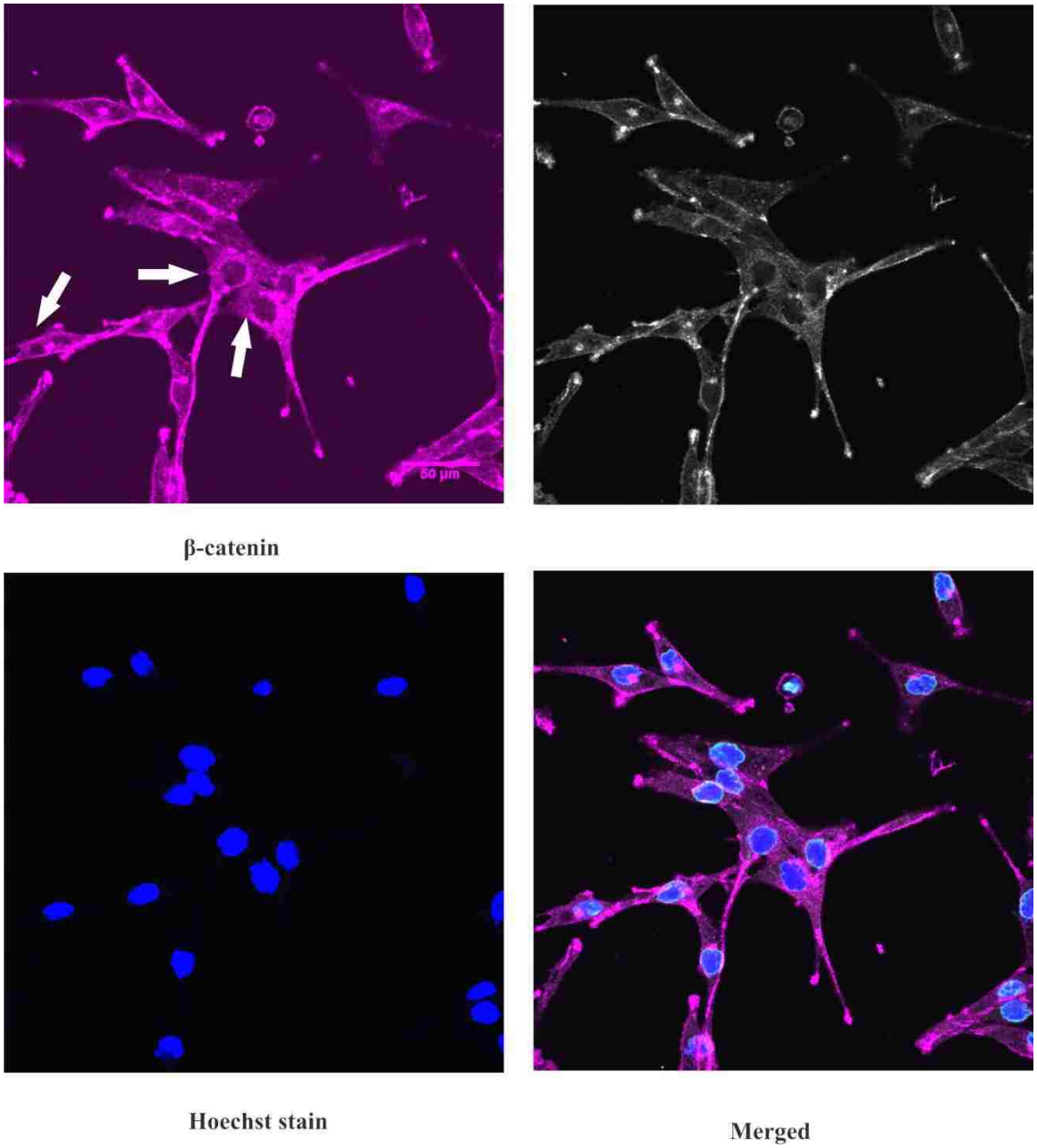


Supplementary figure Ie: NIH/3T3 cells induced with 100 nM insulin for 30 – 60 min post serum starvation

Supplementary figure II

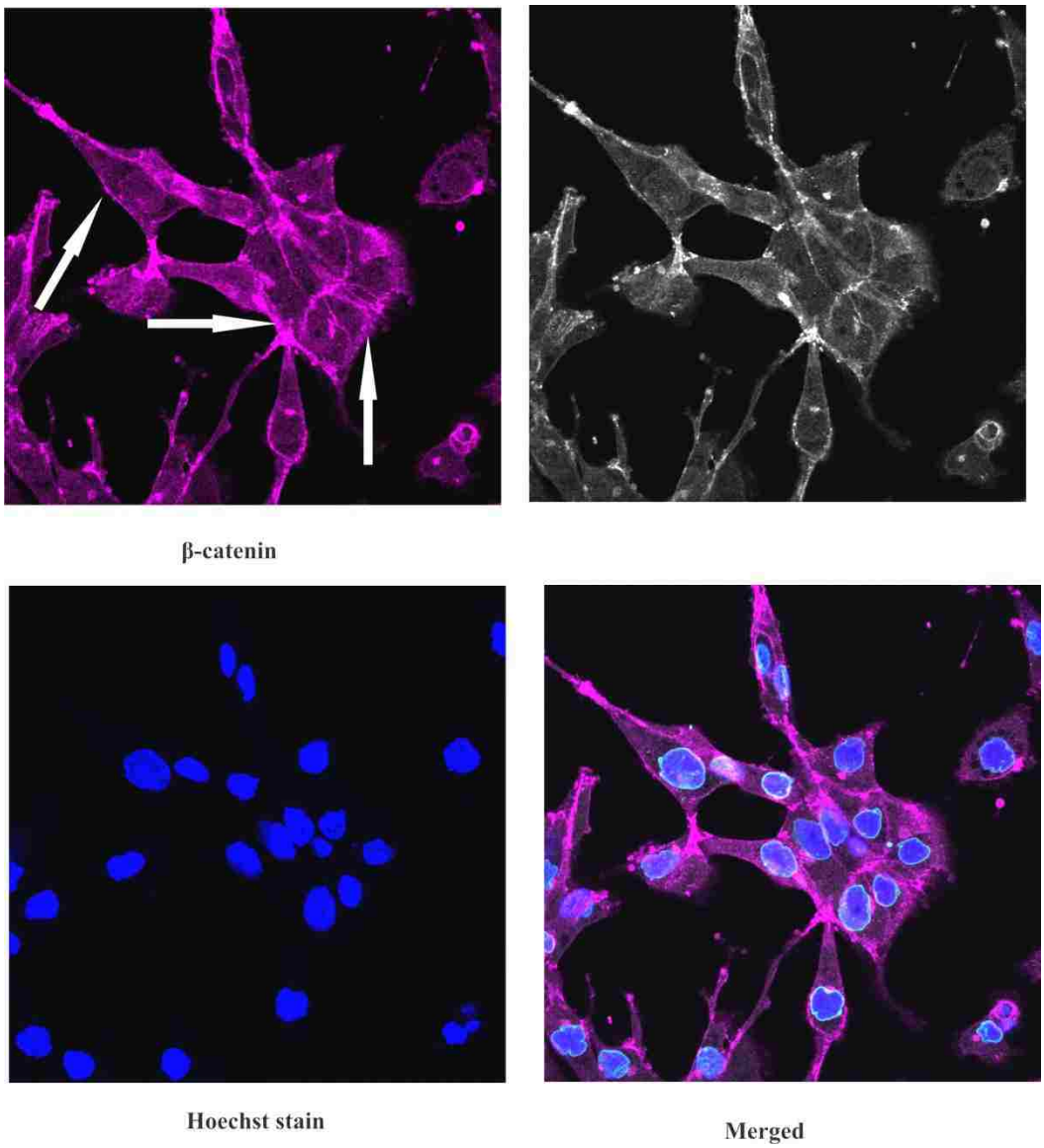
Individual immunoflorescence images corresponding to the fig. 17b from Chapter 5.

A172 control/untreated cells (arrows indicate nuclear β-catenin)



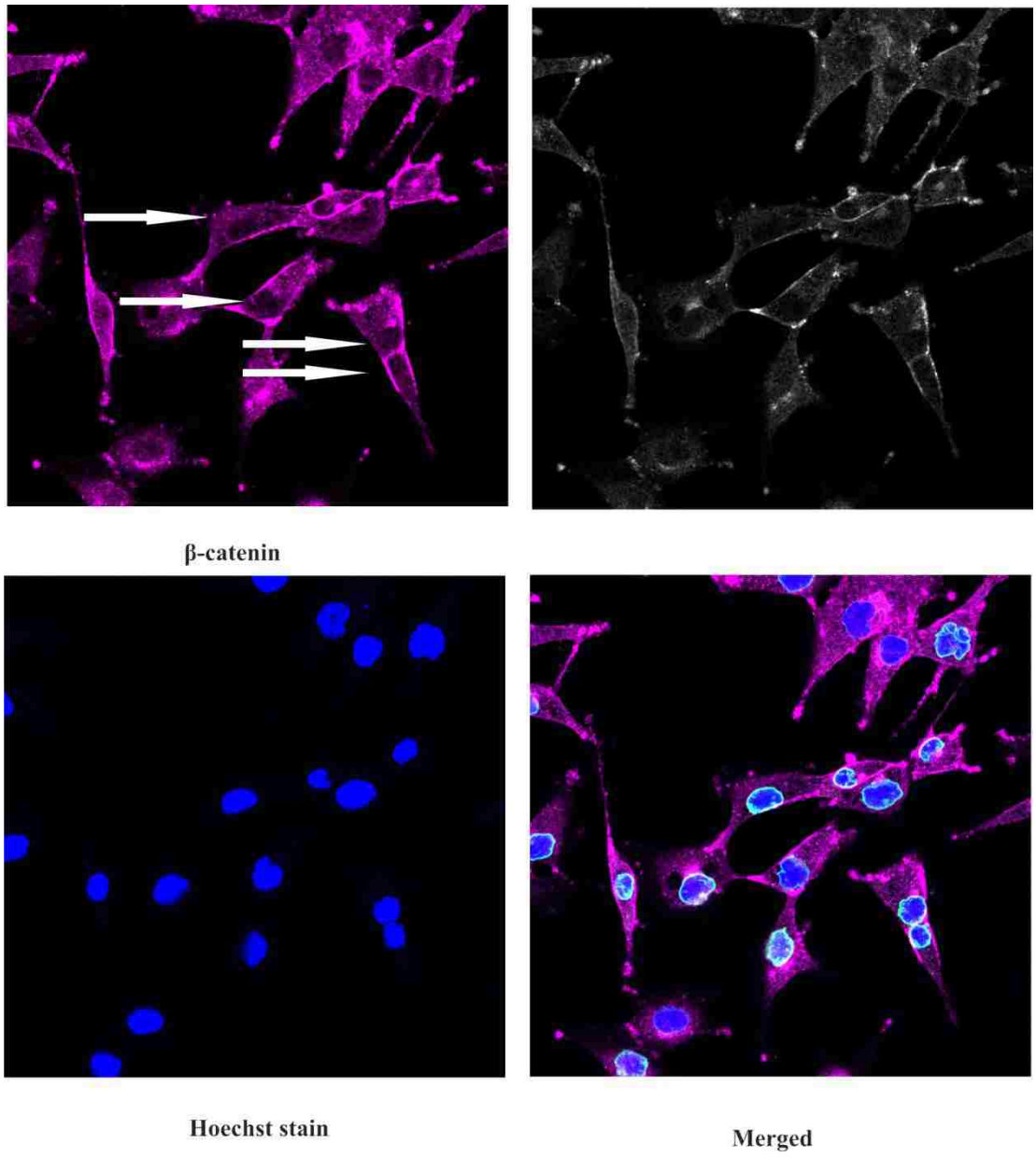
Supplementary figure IIa: A172 cells untreated

A172 cells treated with 20 mM LiCl (arrows indicate profound nuclear localization of β-catenin)



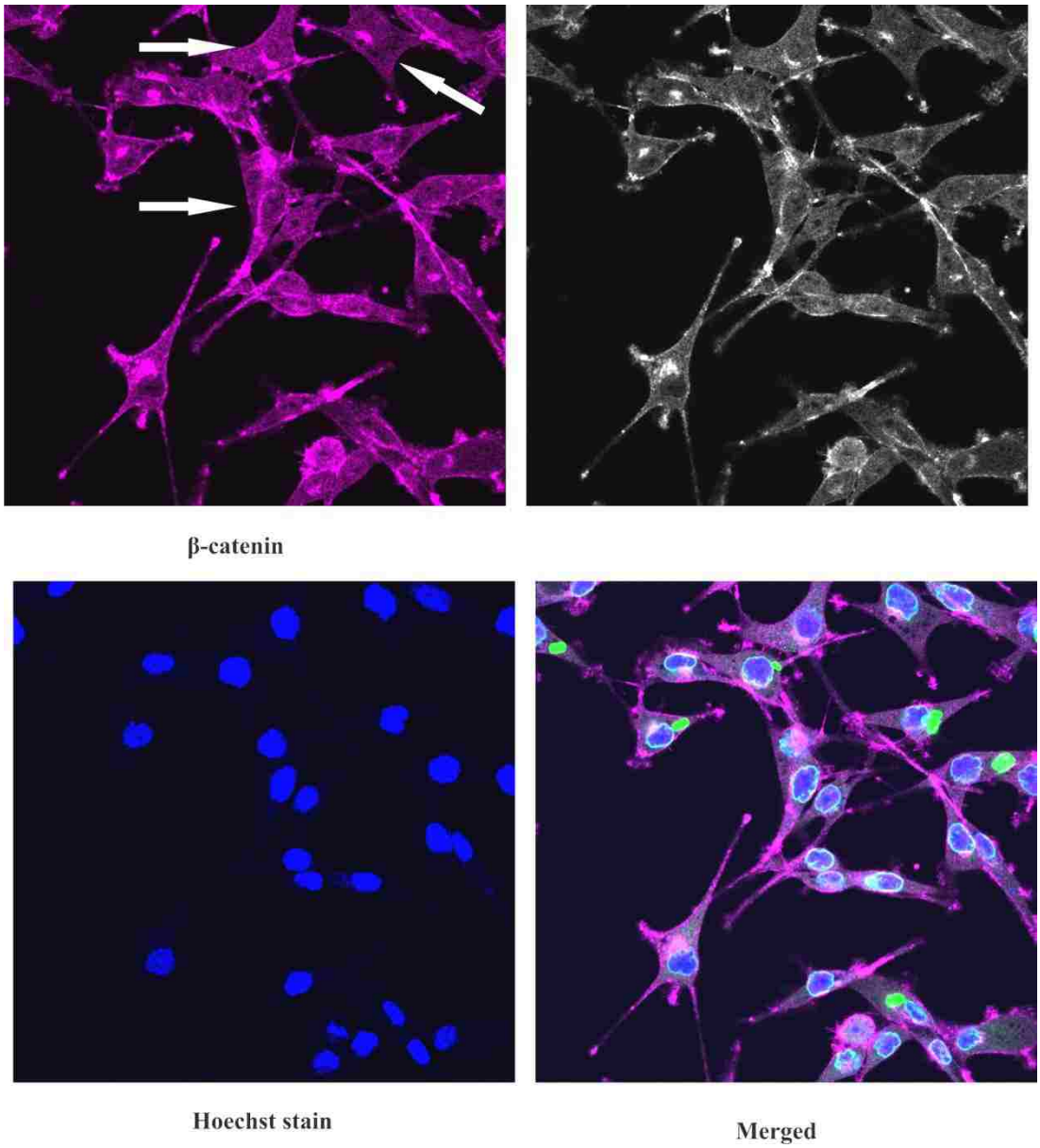
Supplementary figure IIb: A172 cells treated with 20 mM LiCl for 24 hr

A172 cells treated with 100 µM $BeSO_4$ (arrows indicate minimal nuclear localization of β-catenin)



Supplementary figure IIc: A172 cells treated with 100 µM $BeSO_4$ for 24 hr

A172 cells treated with 20 µM SB216763 (arrows indicate prominent nuclear localization of β-catenin)

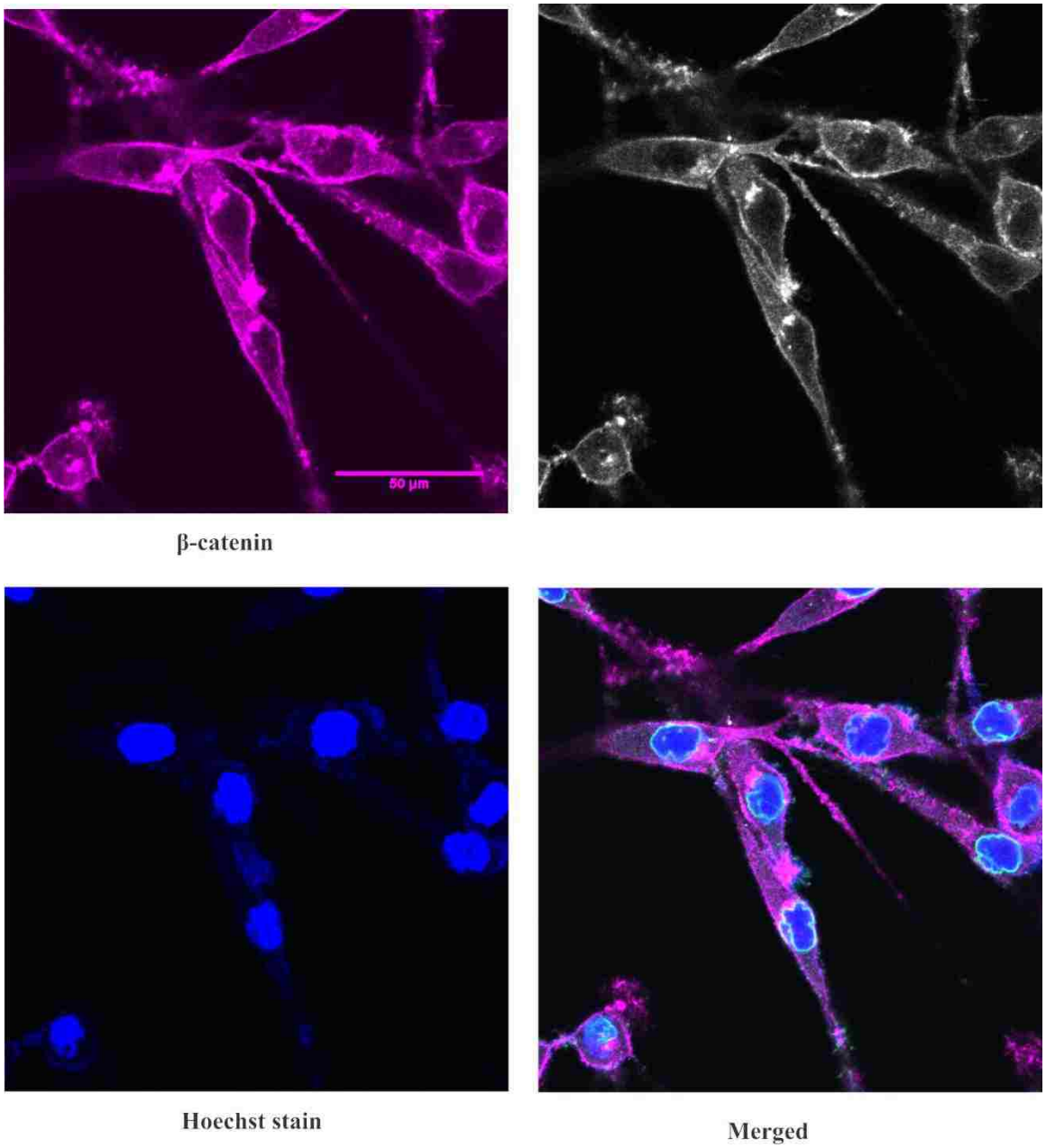


Supplementary figure IId: A172 cells treated with 20 µM SB216763 for 24 hr

Supplementary Figure III

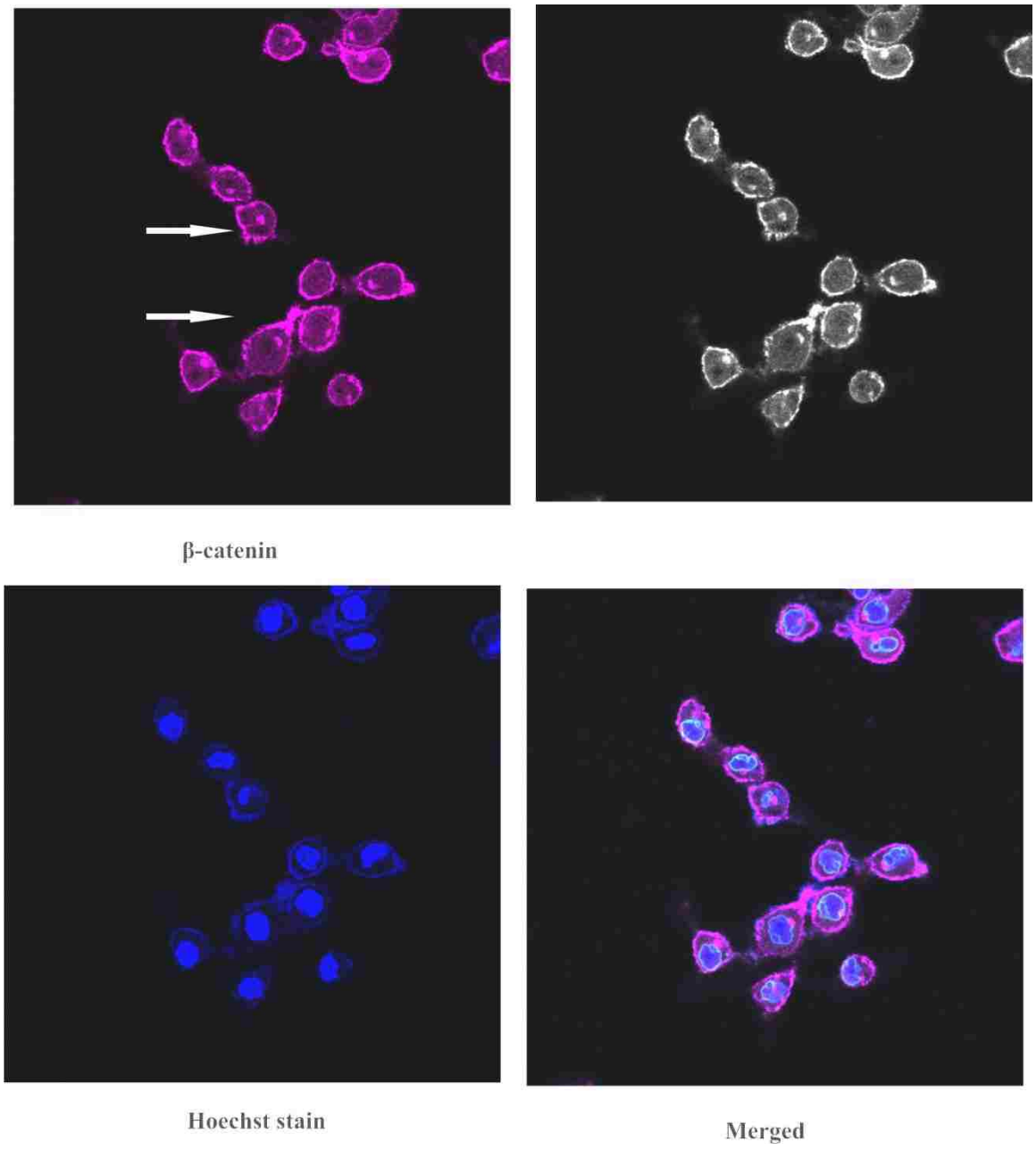
Individual immunoflorescence images corresponding to the fig. 17c from Chapter 5.

A172 P1B control/untreated cells (arrows indicate nuclear β-catenin)



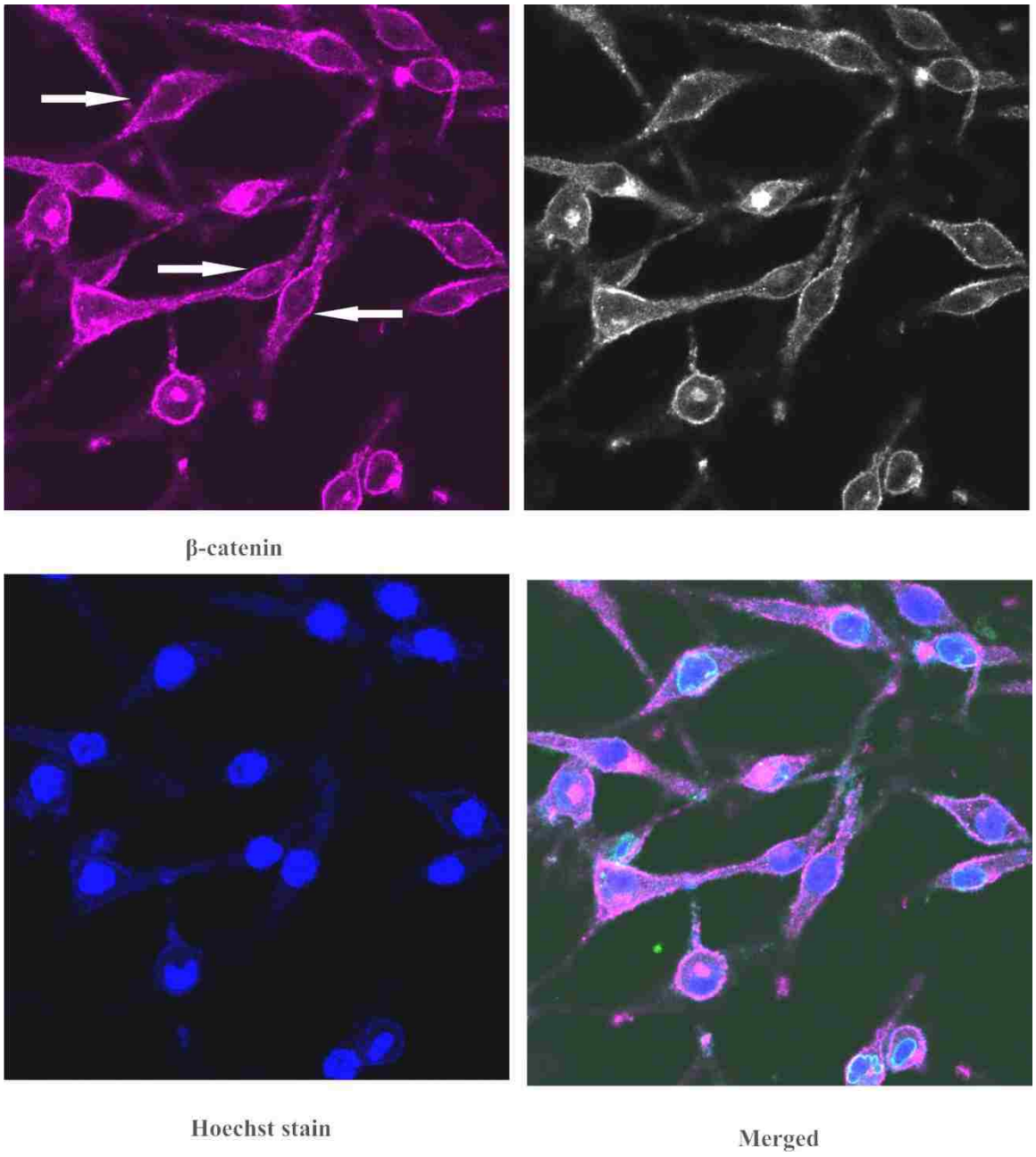
Supplementary figure IIIa: A172 P1B cells untreated

A172 P1B cells treated with 20 mM LiCl (arrows indicate prominent nuclear localization of β-catenin)



Supplementary figure IIIb: A172 P1B cells treated with 20 mM LiCl for 24 hr

A172 P1B cells treated with 100 µM $BeSO_4$ (arrows indicate minimal nuclear localization of β-catenin)



Supplementary figure IIIc: A172 P1B cells treated with 100 µM $BeSO_4$ for 24 hr

APPENDIX I

Statistical analysis

Table 9. In vitro kinase activity of purified recombinant GSK-3β

Inhibitor	**Relative specific activity (GSK-3β)**	***P* – value**	**Significantly different (unpaired t-test)**
Control	1.00	-	-
0.3 μM Be^{2+}	0.97	*P* >0.05	No
1 μM Be^{2+}	0.62	*P* < 0.005	Yes
3 μM Be^{2+}	0.47	*P* < 0.005	Yes
10 μM Be^{2+}	0.13	*P* < 0.0005	Yes
30 μM Be^{2+}	0.11	*P* < 0.0005	Yes
100 μM Be^{2+}	0.02	*P* < 0.0005	Yes
300 μM Be^{2+}	0.04	*P* < 0.0005	Yes
Control	1.00	-	-
1 mM Li^{+}	0.91	*P* >0.05	No
3 mM Li^{+}	0.75	*P* < 0.005	Yes
10 mM Li^{+}	0.51	*P* < 0.05	Yes
30 mM Li^{+}	0.23	*P* < 0.0001	Yes
100 mM Li^{+}	0.01	*P* < 0.0001	Yes
300 mM Li^{+}	-0.03	*P* < 0.0001	Yes

Table 9. Two tailed unpaired t-test was used to compare the *in vitro* GSK-3β activity of the samples treated with inhibitors against their corresponding control. (Data from same experiment)

Table 10. In vitro kinase activity of pure recombinant PKA

Inhibitor	**Relative specific activity (PKA)**	***P* – value**	**Significantly different (unpaired t-test)**
Control	1.00	-	-
0.3 µM Be^{2+}	0.67	$P > 0.05$	No
1 µM Be^{2+}	0.63	$P < 0.005$	Yes
3 µM Be^{2+}	0.70	$P < 0.05$	Yes
10 µM Be^{2+}	0.69	$P < 0.05$	Yes
30 µM Be^{2+}	0.40	$P < 0.0001$	Yes
100 µM Be^{2+}	0.32	$P < 0.0001$	Yes
300 µM Be^{2+}	0.31	$P < 0.0001$	Yes
Control	1.00	-	-
1 mM Li^{+}	0.77	$P < 0.05$	Yes
3 mM Li^{+}	0.88	$P > 0.05$	No
10 mM Li^{+}	0.85	$P > 0.05$	No
30 mM Li^{+}	0..99	$P > 0.05$	No
100 mM Li^{+}	0.73	$P < 0.05$	Yes
300 mM Li^{+}	0.31	$P < 0.0001$	Yes

Table 10. Two tailed unpaired t-test was used to compare the *in vitro* PKA activity of the samples treated with inhibitors against their corresponding control. (Data from same experiment)

Table 11 Validation of the z-lyte assay using PKA inhibitor H89

Inhibitor (H89)	**Relative specific activity (GSK-3β)**	***P* – value**	**Significantly different (unpaired t-test)**	**Relative specific activity (PKA)**	***P* – value**	**Significantly different (unpaired t-test)**
0 µM	1.00	-	-	1.00	-	-
0.1 µM	1.00	*P* >0.05	No	0.57	*P* < 0.0005	Yes
0.3 µM	1.03	*P* >0.05	No	0.32	*P* < 0.0001	Yes
1 µM	0.99	*P* >0.05	No	0.125	*P* < 0.0001	Yes
3 µM	0.99	*P* >0.05	No	0.05	*P* < 0.0001	Yes
10 µM	0.94	*P* >0.05	No	0.01	*P* < 0.0001	Yes

Table 11. Two tailed unpaired t-test was used to compare the *in vitro* PKA activity of the samples treated H89 against their corresponding control (data from same experiment). Two tailed unpaired t-test was used to compare the *in vitro* GSK-3β activity of the samples treated H89 against their corresponding control (data from same experiment).

Table 12. TR-FRET assay to quantify the pSer9-GSK-3β signal

Inhibitor	**Relative TR-Fret signal (GSK-3β)**	***P* – value**	**Significantly different (unpaired- t test)**
Control	1.000	-	-
10 μM Be^{2+}	1.002	*P* >0.05	ns
30 μM Be^{2+}	0.908	*P* >0.05	ns
100 μM Be^{2+}	1.037	*P* >0.05	ns
Control	1.00	-	-
10 mM Li^{+}	1.27	*P* >0.05	ns
20 mM Li^{+}	1.54	*P* <0.05	significant
30 mM Li^{+}	1.92	ns	ns

Table 12. Two tailed unpaired t-test was used to compare the TR-FRET signal of the inhibitors treated cells with their corresponding control cells (data from same experiment).

Table 13. Flow analysis of nuclear β-catenin in stable nuclei

Treatment	Duration	Mean Fluorescence / nuclei (% of control +/- std dev)
control	24 hr	100 (+/- 5)
10 μM $BeSO_4$	24 hr	110 (+/- 3)
30 μM $BeSO_4$	24 hr	148 (+/- 5)
100 μM $BeSO_4$	24 hr	151 (+/- 12)
10 mM LiCl	24 hr	150 (+/- 12)
20 mM LiCl	24 hr	188 (+/- 23)
30 mM LiCl	24 hr	180 (+/- 20)

Table 13 Mean fluorescence values corresponding to the nuclear β-catenin. Stable nuclei isolated from A172 cells treated with inhibitors for 24 hr.

Table 14. Flow analysis of nuclear p53 in stable nuclei

Treatment	Duration	Mean Fluorescence / nuclei (% of control +/- std dev)
control	24 hr	100 (+/- 3)
100 μM $BeSO_4$	24 hr	124 (+/- 5)
20 mM LiCl	24 hr	148 (+/- 1)

Table 14 Mean fluorescence values corresponding to the nuclear p53. Stable nuclei isolated from A172 cells treated with inhibitors for 24 hr.

Appendix II

Physiologically relevant concentration of lithium is more cytotoxic to cells compared to beryllium at 24 hr

Introduction

The IC_{50} value of lithium = 12 mM and the IC_{50} value of beryllium is ~2 µM. When A172 cells are cultured in $BeSO_4$ or LiCl at concentrations close to their IC_{50} values, lithium was found to be more cytotoxic. As observed in the viability test, Li^+ at concentrations close to its IC_{50} was more toxic to the viability of A172 cells (Fig. 4).

Experiment 1

A172 cells were cultured in the presence of $BeSO_4$ and LiCl for 24, 48 or 72 hr. The effect of the inhibitors on the viability of the cells was analyzed using a live cell protease based fluorescence assay. The viability assay shows that Be^{2+} is less cytotoxic to A172 cells even at concentrations way higher than its IC_{50}. Be^{2+} at 100 µM is fairly tolerated by A172 cells even for longer periods of time (Fig 1). There is no big difference in the number of dead cells between the control and beryllium samples. Li^+ treatment on the other hand seems to be causing an increase in cytotoxicity especially at 48 or 72 hr.

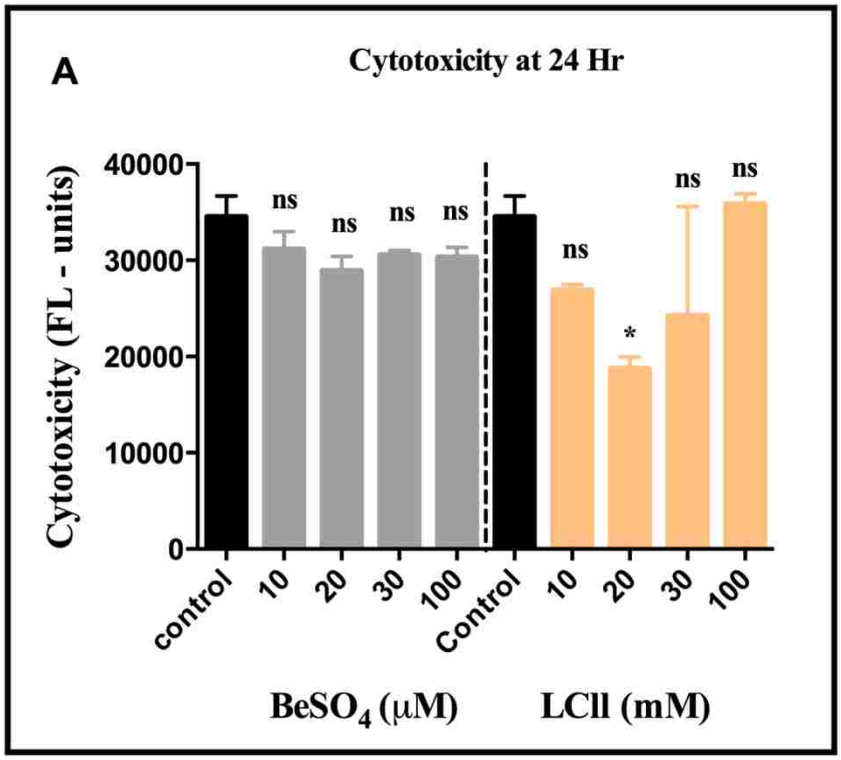


Fig. 1a Effect of $BeSO_4$ and LiCl treatment on cells cytotoxicity at 24 hr. A172 cells were treated with 0, 10, 20, 30, 100 μM $BeSO_4$ or 10, 20, 30, 100 mM LiCl for 24 hr. Cell cytotoxicity was assessed using a fluorogenic substrate that can be cleaved by a protease associated with dead cells only. Mean intensities (+/- std. dev) are reported here. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significant) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

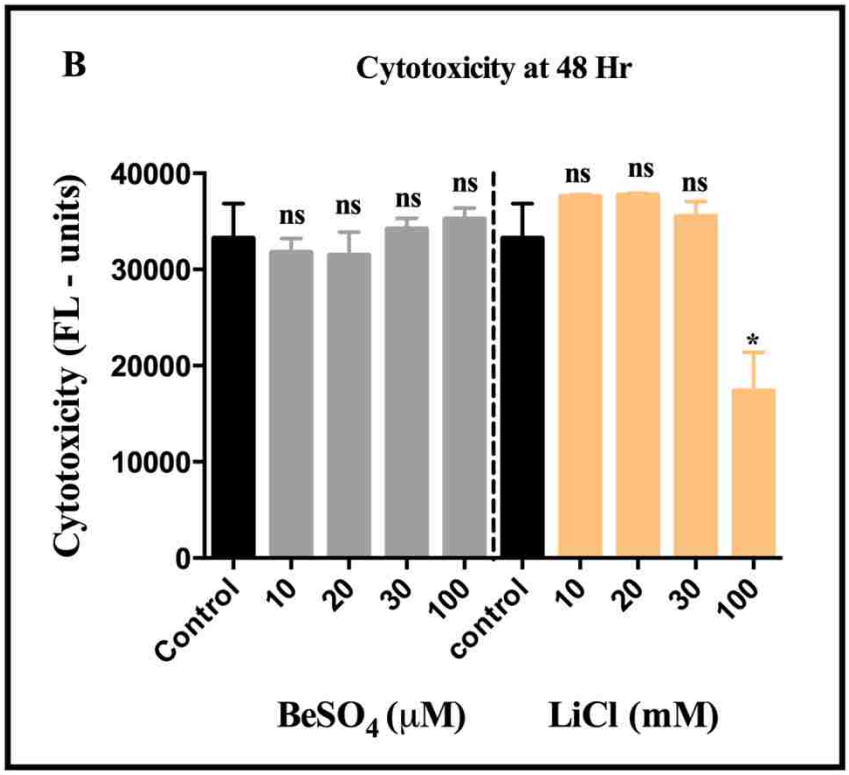


Fig. 1b Effect of $BeSO_4$ and LiCl treatment on cells cytotoxicity at 48 hr. A172 cells were treated with 0, 10, 20, 30, 100 μM $BeSO_4$ or 10, 20, 30, 100 mM LiCl for 48 hr. Cell cytotoxicity was assessed using a fluorogenic substrate that can be cleaved by a protease associated with dead cells only. Mean intensities (+/- std. dev) are reported here. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significant) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

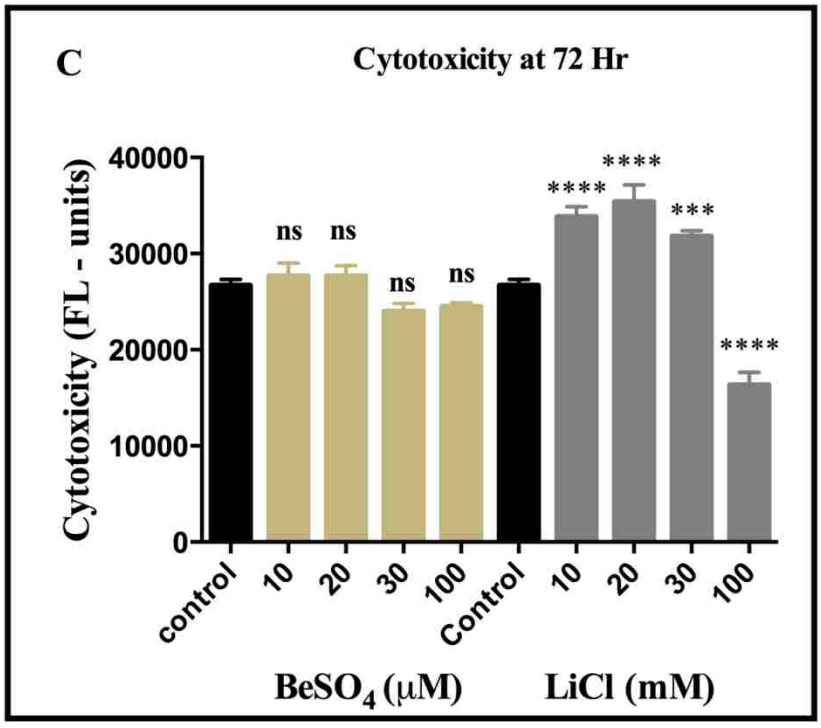


Fig. 1c Effect of $BeSO_4$ and LiCl treatment on cells cytotoxicity at 72 hr. A172 cells were treated with 0, 10, 20, 30, 100 μM $BeSO_4$ or 10, 20, 30, 100 mM LiCl for 72 hr. Cell cytotoxicity was assessed using a fluorogenic substrate that can be cleaved by a protease associated with dead cells only. Mean intensities (+/- std. dev) are reported here. Data was analyzed using one-way ANOVA ($P < 0.05$ considered significant) by Tukey's multiple comparison test. GraphPad Prism trial version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

Experiment 2

A172 cells were treated with $BeSO_4$ or LiCl for 24 hr. Bright field images of the A172 cells were captured using Nikon Inverted Microscope Eclipse TE2000-U (dia-illuminator 100W). When treated with 10-100 μM $BesO_4$ for 24 hr no major morphological changes were observed in A172 cells (Fig 2.1). When A172 cells are cultured in the presence of LiCl for 24 hr a marked change in the morphological appearance of the cells was observed (Fig. 2.2).

Results

Images of the A172 cells cultured in the presence of $BeSO_4$ for 24 hr, shows that LiCl is more toxic to cells compared to $BeSO_4$ at concentrations similar to their IC_{50} values. A172 cells seem to be tolerating LiCl at a concentration of 10 mM (Fig 2.2b). When the LiCl concentration was increased to 20 mM or 30 mM it induced high cytotoxicity in A172 cells (Fig. 2.2 c&d). LiCl at 100 mM concentration was extrememly toxic to A172 cells at 24 hr.

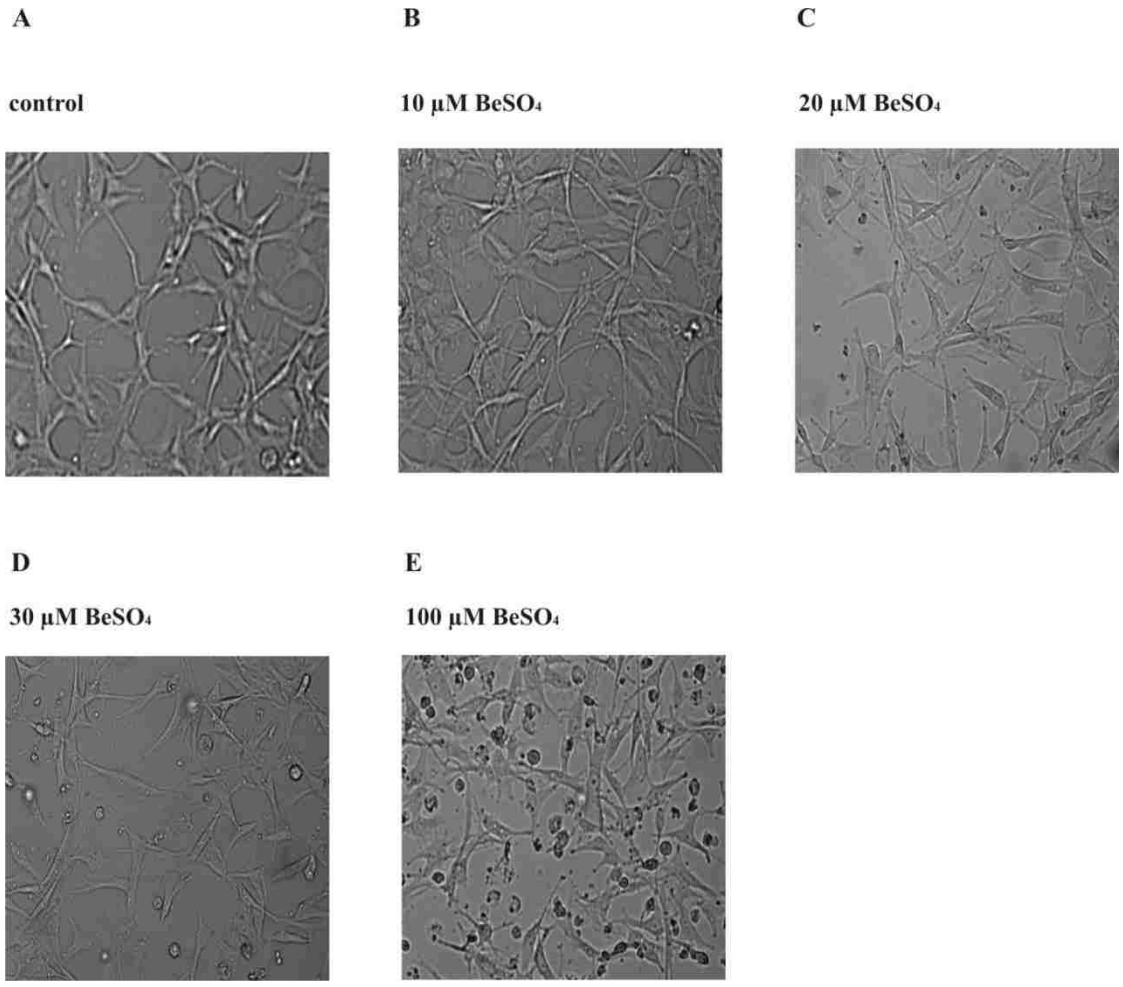


Fig 2.1Be^{2+} doesn't produce any extreme morphological changes in A172 cells at 24 hr. A172 cells were cultured in the presence of 0, 10, 20, 30 or 100 µM $BeSO_4$ for 24 hr.

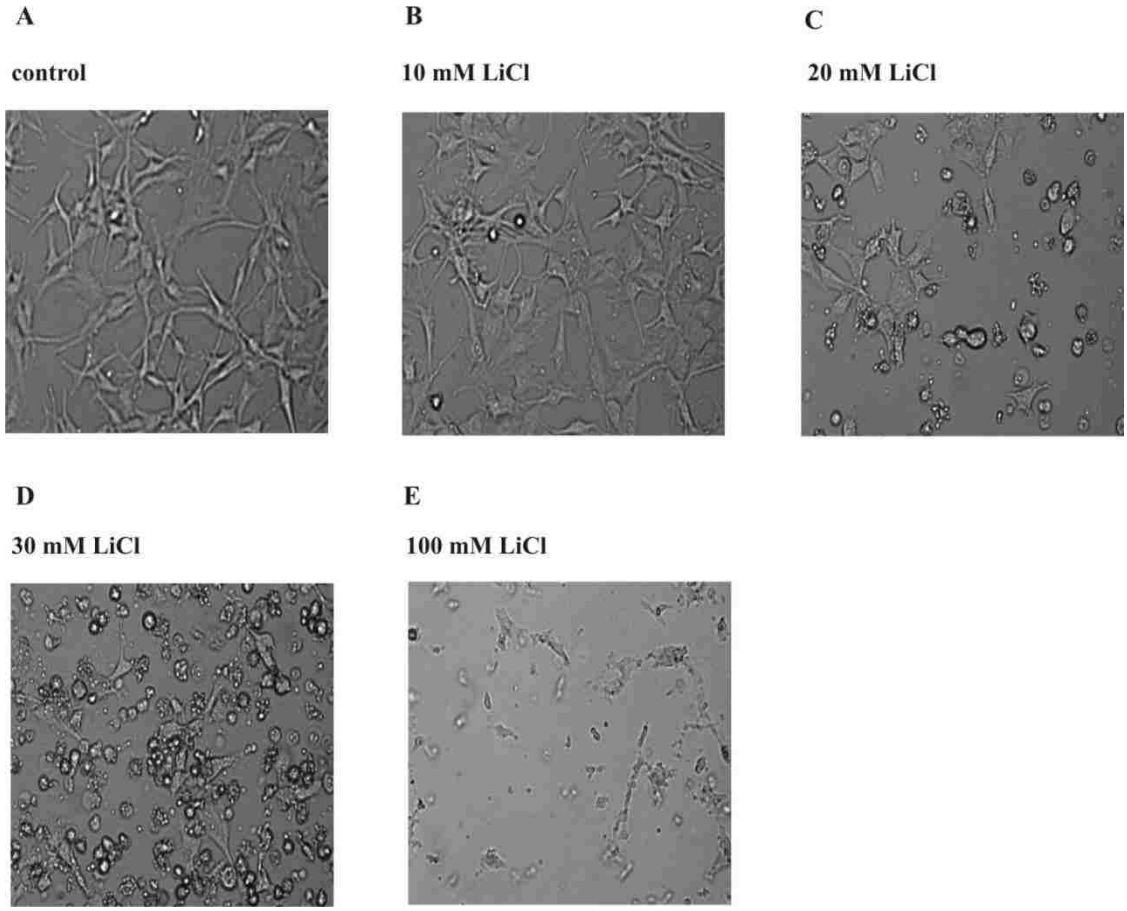


Fig 2.2 Li^+ treatment leads to extreme morphological changes in A172 cells at 24 hr. A172 cells were cultured in the presence of 0, 10, 20, 30 or 100 mM LiCl for 24 hr.

Appendix III

Quatitative analysis of Ser-9 phsophorylation of GSK-3β in A172 cells cultured in the presence of $BeSO_4$ or LiCl at 48 hr

Introduction

Li^+ caused an increase in the Ser-9 phosphorylation of GSK-3β in A172 cells at 24 hr. However Be^{2+} did not induce any big change in the Ser-9 phosphorylation of GSK-3β. Here we have analyzed the effect of $BeSO_4$ or LiCl on the Ser-9 phosphorylation of GSK-3β of A172 cells at 48 hr via flow cytometry.

Flow cytometry

Cells were treated with $BeSO_4$ and LiCl for 48 hr. After the treatment period, cells were collected by trypsinization, washed with PBS and fixed with 4% paraformal dehyde. Cells were then permeabilized with methanol. Cells were blocked with starting block TBS (prod#37543, Thermo Scientific), supplemented with Halt protease and phosphatase inhibitor cocktail (Pierce cat#78442). Fixed and blocked cells were then incubated with pSer9-GSK-3β rabbit monoclonal antibody (clone D85E12, cat#5558, Cell Signaling Technology) over night at 4℃, followed by FITC conjugated goat anti-rabbit secondary antibody (cat#sc-2012, Santa Cruz Biotechnology) at 0.5 µg/1x10^6 cells. Cells were run on a BD FACSCalibur flow cytometer recording 20,000 events per sample, and FITC channel fluorescence intensity was analyzed using CellQuest Pro Software.

Results

At 48 hr beryllium did not induce any clear increase in the Ser-9 phosphorylation of GSK-3β (Fig. 1a). Lithium treatment at 48 hr caused a concentration dependent increase in the Ser-9 phosphorylation of GSK-3β.

Conclusion

This study shows that even at longer exposure time i.e. 48 hr, beryllium has little effect on the Ser-9 phosphorylation of GSK-3β.

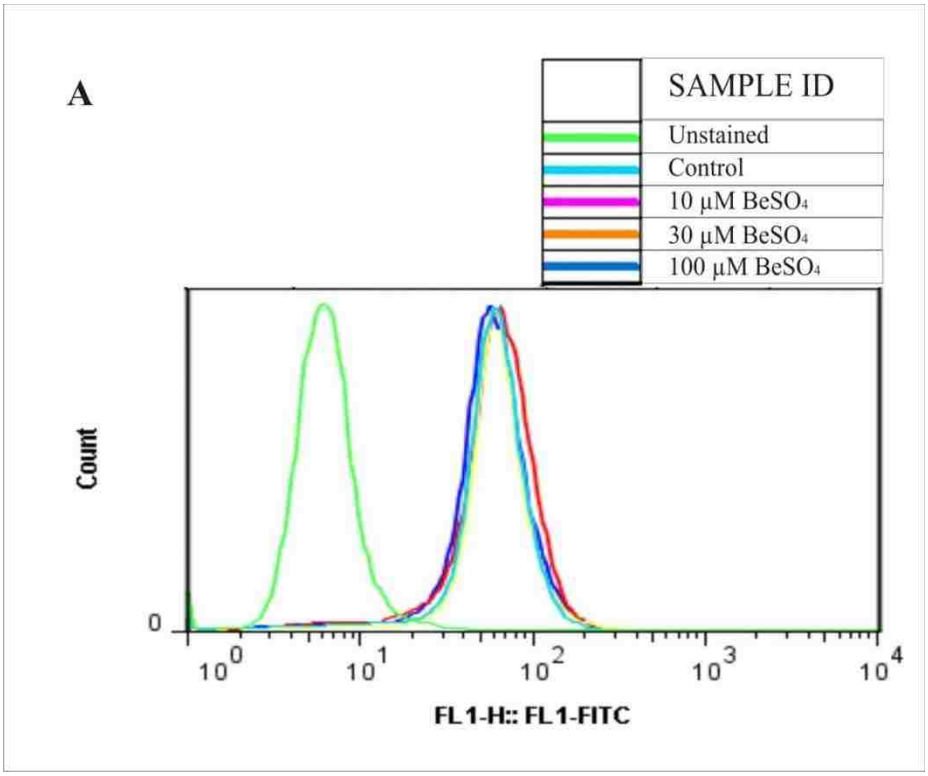


Fig. 1a Flow cytometric analysis of the Be^{2+} treatment effect on Ser-9 phosphorylation of GSK-3β at 48 hr. A172 cells were treated with 0, 10, 30, 100 $BeSO_4$ for 48 hr. The change in the pSer-9 status of GSK-3β was assessed using a pSer9-GSK3β primary antibody and FITC conjugated secondary antibody. Each flow cytometry histograms represents the mean fluorescence per cell value obtained from independent replicates for each inhibitor used at different concentrations.

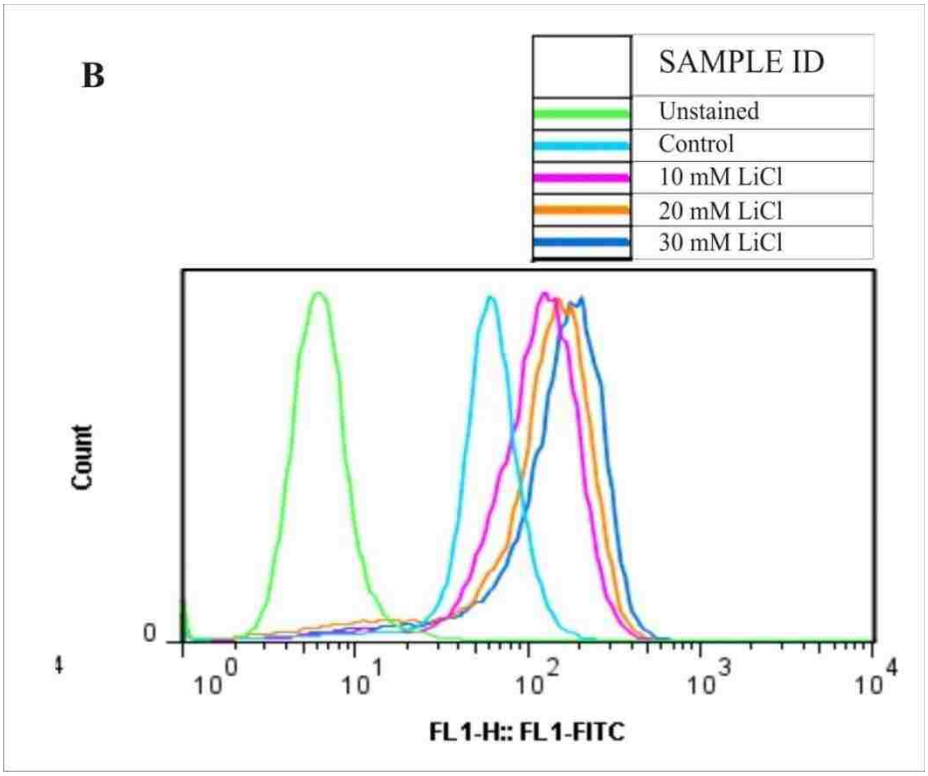


Fig. 1b Flowcytometric analysis of the Li^+ treatment effect on Ser-9 phosphorylation of GSK-3β at 48 hr. A172 cells were treated with 0, 10, 20, 30 mM LiCl for 48 hr. The change in the pSer-9 status of GSK-3β was assessed using a pSer9-GSK3β primary antibody and FITC conjugated secondary antibody. Each flow cytometry histograms represents the mean fluorescence per cell value obtained from independent replicates for each inhibitor used at different concentrations.

Appendix IV

Measuring beryllium induced up regulation of p53 expression using nuclei flow analysis

Introduction

To confirm that effect of $BeSO_4$ on nuclear β-catenin, flow-analysis of nuclei isolated from A172 cells treated with $BeSO_4$ or LiCl was used. Along with the β-catenin analysis, the p53 protein levels were also tested independently in the nuclei isolated from A172 cells treated with $BeSO_4$ or LiCl. The aim of this experiment was to validate the fact that nuclei flow analysis method can be used to quantify protein expression.

Result

The flow analysis of nuclei isolated from A172 cells treated with $BeSO_4$ or LiCl for 24 hr indicate an increase in p53 levels. Beryllium treatment caused an increase in the p53 protein levels in A172 and HFL cells (Gorjala and Gary, 2010; Mudireddy et al., 2014). The nuclei flow-analysis results confirm the beryllium induced upregulation of p53 expression.

Discussion

The flow analysis could be used to quantify the Be^{2+} induced upregulation of p53. The ability of the flow analysis technique to successfully detect the increase in the p53 level, validates it as a reliable quantitative method to detect the up regulation or down regulation of proteins.

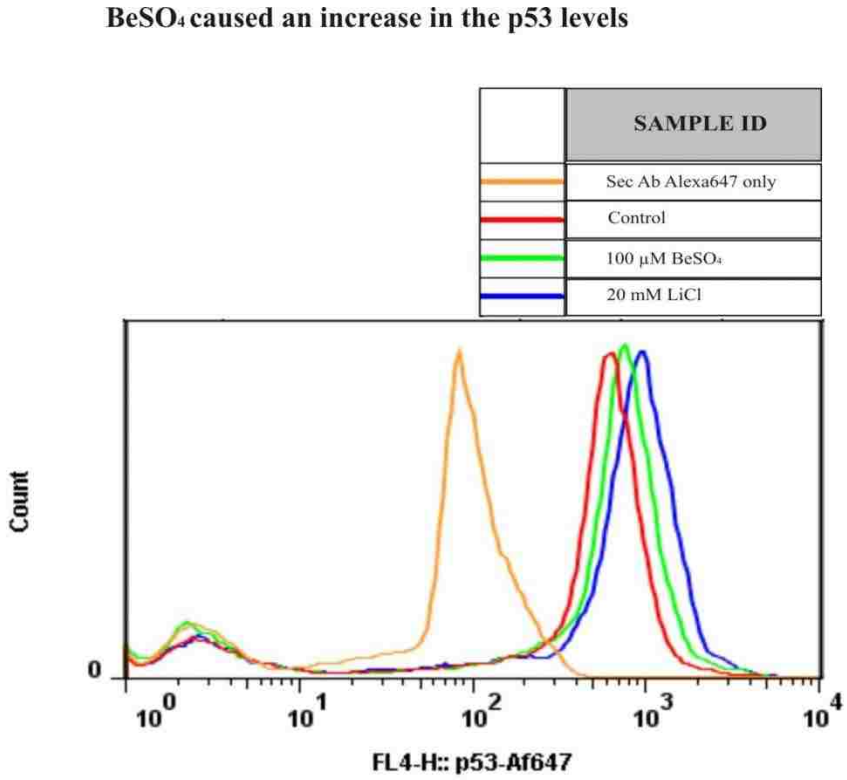


Fig. 1 Detection of increase in p53 level after beryllium treatment at 24 hr in A172 cells. A172 cells were treated with 0, 100 µM $BeSO_4$ or 20 mM LiCl for 24 hr. Stable nuclei were extracted from the Be^{2+} or Li^{+} treated cells. The effect of Be^{2+} or Li^{+} on the nuclear localization of p53 was assessed using a mouse mono clonal p53 primary antibody and an Alexa647 – conjugated secondary antibody. For each treatment, mean fluorescence per cell was determined from independent replicates and representative peak for each treatment is shown here.

Appendix V

Validation of A172 cells over expressing wt GSK-3β, GSK-3β S9A and GSK-3β K85A

Introduction

Mammalian cells over expressing wt GSK-3β and its mutants can serve as valuable tools in our study to characterize beryllium as a potent GSK-3β-inhibitor. Over expression of GSK-3β in A172 cells will provide a system with abundant amont of substrate protein to analyze the effect of inhibitors. A172 cells were transfected with the addgene plasmid#14753 – HA GSK-3β wt pcDNA3, plasmid#14754 – HA GSK-3β S9A pcDNA3, plasmid#14755 – HA GSK-3β K85A pcDNA3 as explained in the materials and methods section.

Results

Fig. 1 The total cell lysates extracted from these clones along with proper controls were probed for the expression of "HA – tag" using a rabbit polyclonal anti HA antibody (Thermoscientific #CAB3872).

P1B, P1C, P1D = A172 clones over expressing wt GSK-3β,

P2A, P2B, P2C = A172 clones over expressing GSK-3β S9A mutant

P3A, P3B = A172 over expressing GSK-3β K85A mutant

The detection of HA band in the transfected A172 cells validates the success of transfection, since only A172 cells tranfected with the addgene clones will be expressing the HA-tag.

Discussion

Fig. 1 Expression of HA-tag in the A172 cells transfected with the addgene clones is observed indicating a successful integration and expression of the addgene plasmids in the host cells. The clones demonstrating the maximum expression of the HA-tag were selected for further analysis. The selected clones are:

A172 clones over expressing wt GSK-3β – P1B

A172 clones over expressing GSK-3β S9A mutant – P2B

A172 over expressing GSK-3β K85A mutant – P3B

The P1B clone (A172 clones over expressing wt GSK-3β) was used in the confocal imaging experiment to demonstrate the effect of beryllium on the nuclear localization of β-catenin.

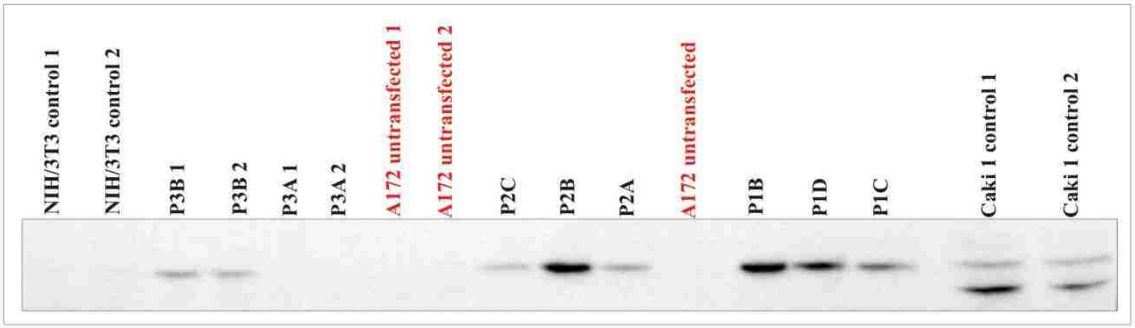


Fig. 1 Western analysis of A172 clones expression wt GSK-3β and its variants. Total cell lysates from the A172 clones expressing various variants of GSK-3β were assessed for the expression of the "HA – tag" using a rabbit polyclonal anti HA antibody. The "date label" refers to the day the samples were extracted. Untransfected A172 along with NIH/3T3 and Caki-1cells serve the purpose of negative controls.

Appendix VI

DRC analysis to investigate the effect of SB216763 on NIH/3T3, A172 and Caki-1 cells

Introduction

SB216763 is a strong GSK-3β inhibitor (Coughlan et al., 2000). To determine the concentration suited best for inducing optimum results in different cell lines a dose response curve experiment was done using A172, NIH/3T3 and Caki-1 cells.

Materials and methods

Cell culture media

RPMI 1640 HEPES (cat#23400-021, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic-mycotic was used for culturing B 35, C6, Caki-1, IMR 32 and SF539 cell lines. Only for NIH/3T3 cells, RPMI 1640 was supplemented with 10% bovine growth serum (BGS) instead of FBS.

Dose response curve - cell counting

Cells were cultured in 60 mm CELLSTAR cell culture dishes (cat#664 160) at 37℃ using a 5% CO_2 incubator. Cells were dosed with culture media supplemented with appropriate concentrations of $BeSO_4$ and the dosing day was counted as Day 0. On the Day 2 the cells were collected by trypsinization using 0.5 ml trypsin (0.05% Trypsin-EDTA). The cells were collected by adding 2.5 ml of RPMI (2.5 ml RPMI + 0.5 ml trypsin = total 3 ml cell suspension). Cells were counted using a cell counter and 0.5 ml of the total cell suspension from day 2 was added to a new 60 mm culture dish. The cells were allowed to grow till day 4 and were trypsinized again,

counted in a manner similar to day 3. 0.5 ml of cells from the total 3 ml cell suspension from day 4 was added to a new 60 mm dish. The process was repeated again on Day 6 with the exception that the cells were discarded instead of re seeding.

Data analysis

The day 2, day 4 and day 6 cell counts were fitted onto a plot using non-linear regression. The effect of $BeSO_4$ on the different cell lines was represented as % of control on the y-axis and the log values of $BeSO_4$ concentration on the x-axis. GraphPad Prism v6.0c (windows) was used for curve fitting.

Results

Caki-1 cells are the most sensitive to SB216763 compared to A172 and NIH/3T3. A concentration of 10-30 µM seems to be tolerated well by A172 and NIH/3T3 cells. Caki-1 cells are sensitive to SB216763 and seem to be tolerating a concentration of 10-20 µM.

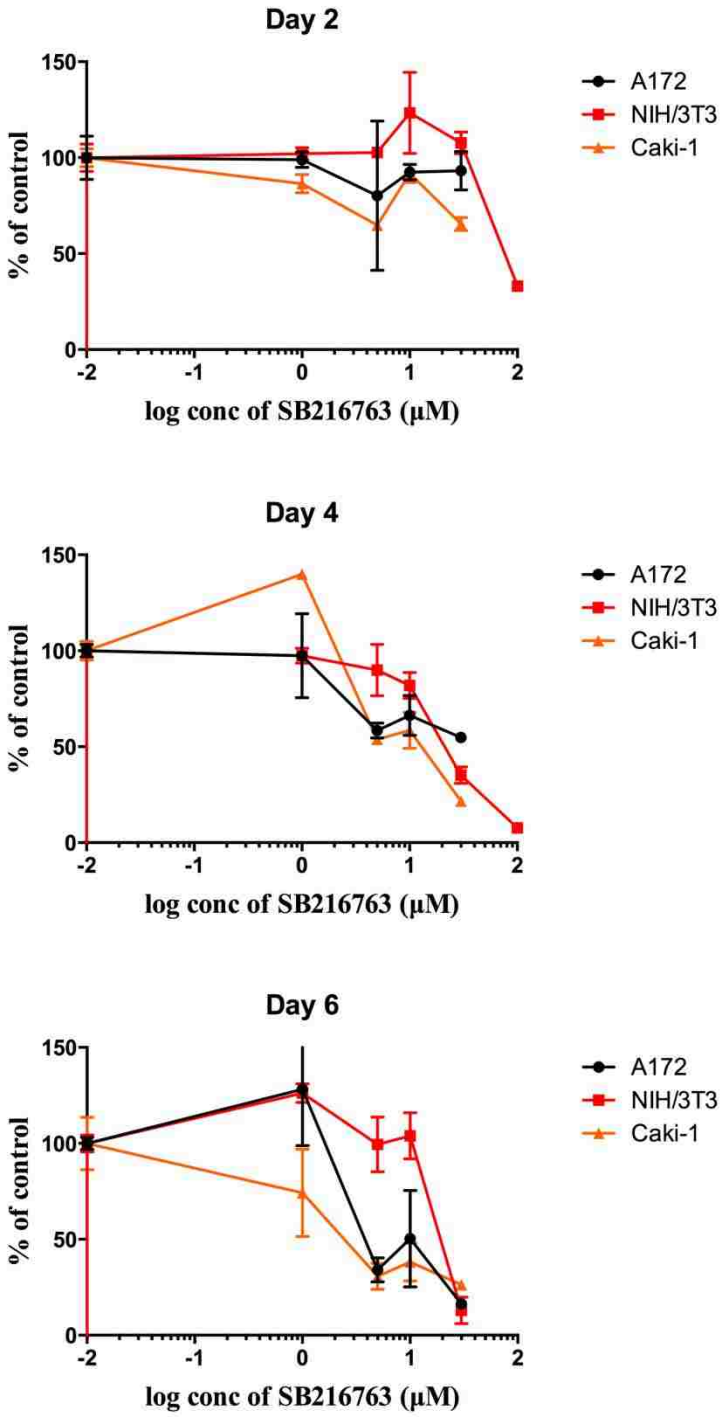


Fig. 1 Dose response curve of A172, NIH/3T3 and Caki-1 cells for SB216763. Caki-1, A172 and NIH/3T3 cells were cultured in the absence or presence of 1, 5, 10, 10 0r 100 µM SB216763 for 24 hr.

Appendix VII

Quantification of phospho-GS bands from fig 15a

Fig 15a Dup I samples pGS band quantification

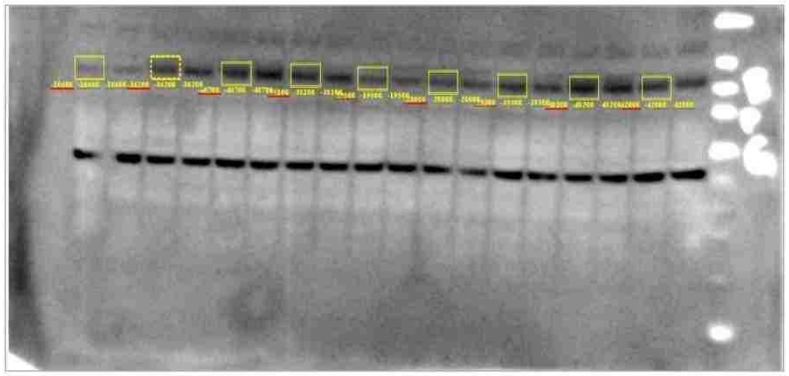
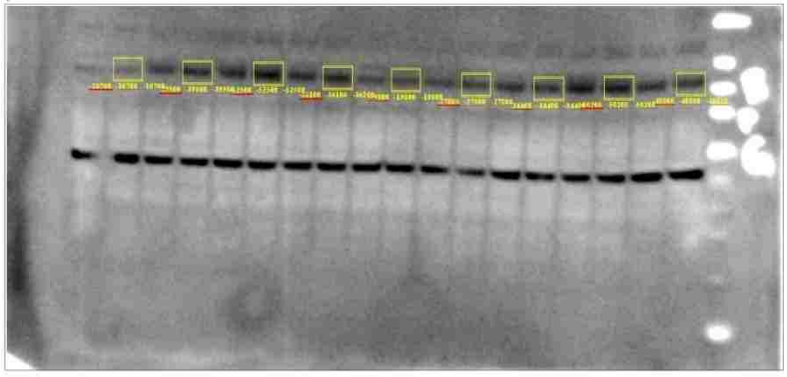


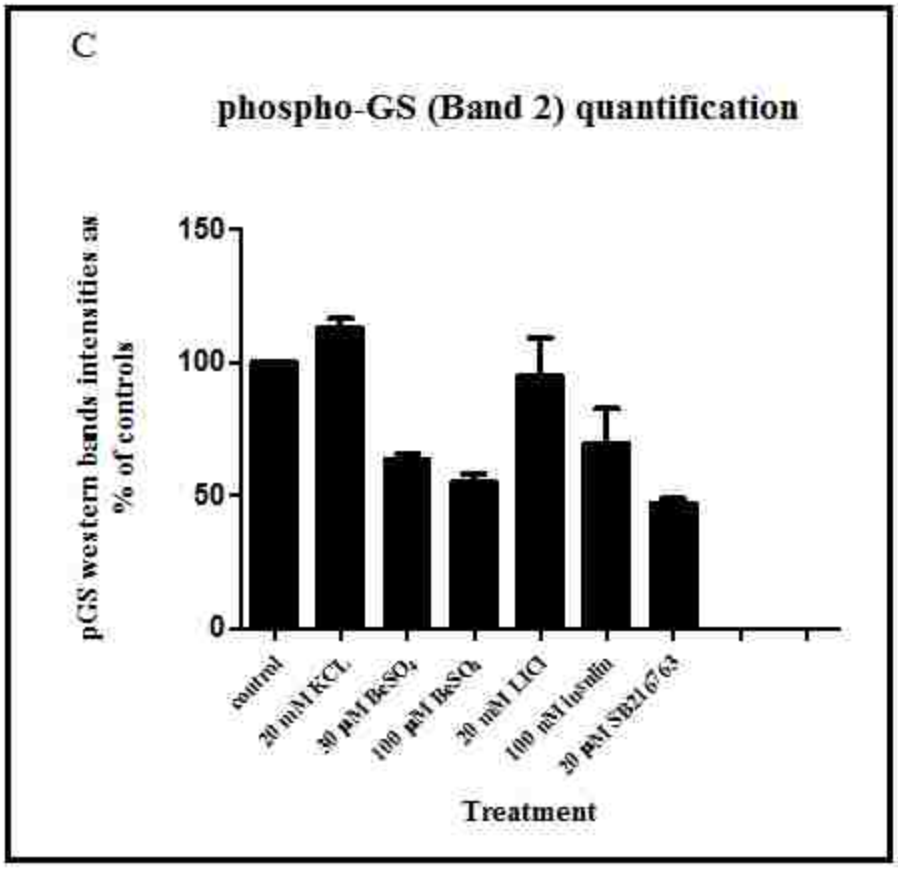
Fig 15a Dup II samples pGS band quantification



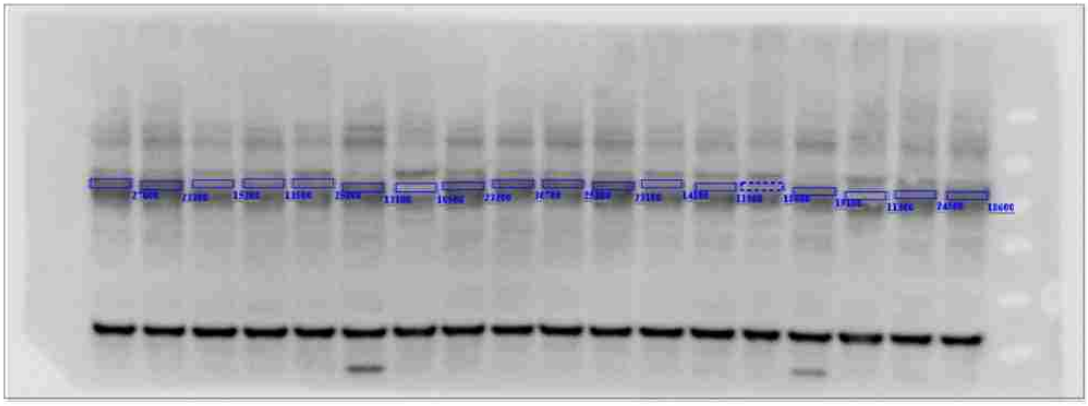
Band	Treatment	Band Intensity background substracted	% of control
1	20 µM SB216763 1	10600	23.55555556
2	20 µM SB216763 2	10700	23.77777778
3	100 nM Insulin 60 min 1	36200	80.44444444
4	100 nM Insulin 60 min 2	39500	87.77777778
5	100 nM Insulin 30 min 1	40700	90.44444444
6	100 nM Insulin 30 min 2	52500	116.6666667
7	100 nM Insulin 15 min 1	38100	84.66666667
8	100 nM Insulin 15 min 2	36100	80.22222222
9	20 mM LiCl 1	19500	43.33333333
10	20 mM LiCl 2	19800	44
11	100 µM $BeSO_4$ 1	28000	62.22222222
12	100 µM $BeSO_4$ 2	27800	61.77777778
13	30 µM $BeSO_4$ 1	39300	87.33333333
14	30 µM $BeSO_4$ 2	34400	76.44444444
15	20 mM KCL 1	48200	107.1111111
16	20 mM KCL 2	50200	111.5555556
17	Control 1	42000	93.33333333
18	Control 2	48000	106.6666667

Appendix VIII

Quantification of pGS bands from fig 16



pGS band quantification of fig 16 image



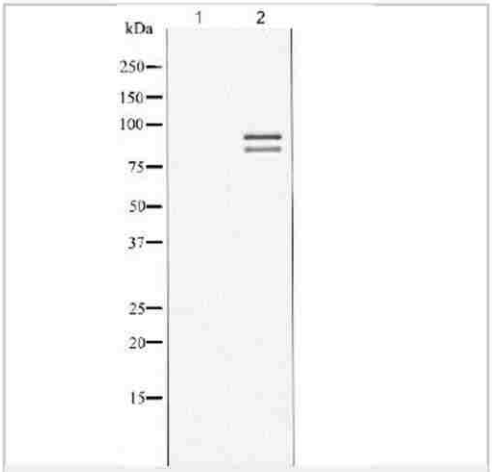
Band	Treatment	Band intensities background substracted	% of controls
1	20 mM KCL 1	27000	116.63067
2	Control 1	23200	100.215983
3	30 uM Be 1	15200	65.6587473
4	100 uM Be 2	13500	58.3153348
5	20 mM Li 1	25300	109.287257
6	100 nM Insulin 1	13100	56.587473
7	SB216763 1	10500	45.3563715
8	10 uM Rott 1	23200	100.215983
9	100 uM CaCl2 1	30700	132.613391
Band	Treatment	Band intensities background substracted	% of controls
10	20 mM KCL 2	25200	108.855292
11	Control 2	23100	99.7840173
12	30 uM Be 2	14100	60.9071274
13	100 uM Be 2	11900	51.4038877
14	20 mM Li 2	18600	80.3455724
15	100 nM Insulin 2	19100	82.5053996
16	SB216763 2	11300	48.812095
17	10 uM Rott 2	24500	105.831533
18	100 uM CaCl2 2	18600	80.3455724

Appendix IX

Expression of glycogen synthase isoforms in NIH/3T3 cells

pGS antibody from AbCam– tested on NIH/3T3 samples

Product **Datasheet**



Western blot - Anti-Glycogen synthase 1 (phospho S645) antibody (ab195743)

All lanes : Anti-Glycogen synthase 1 (phospho S645) antibody (ab195743) at 1/500 dilution

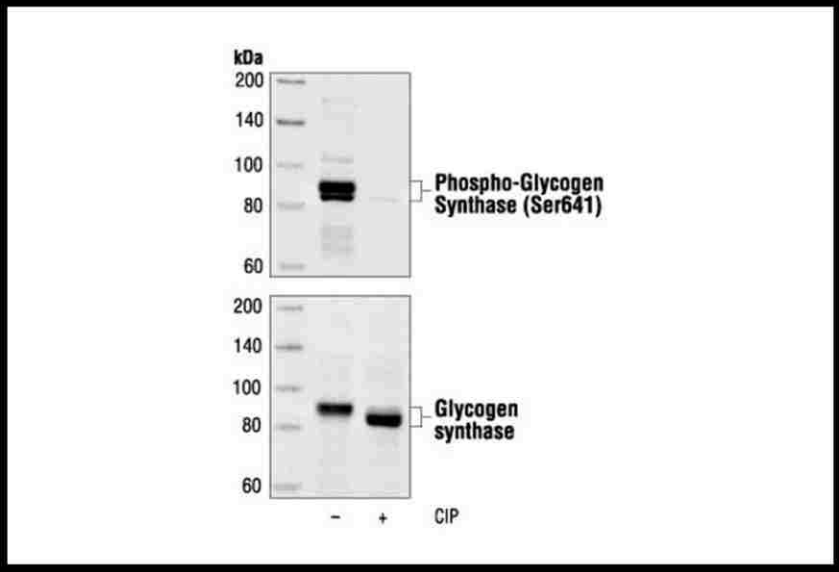
Lane 1 : PMA treated NIH 3T3 whole cell lysate with antigen-specific peptide

Lane 2 : PMA treated NIH 3T3 whole cell lysate

Lysates/proteins at 12.5 µg per lane.

Predicted band size : 84 kDa

pGS antibody from cell signaling technologies – tested on NIH/3T3 samples



Appendix X

Validation of FRET-based kinase assay

To validate the FRET-based kinase assay, purified recombinant GSK-3β and purified recombinant PKA (protein kinase A) proteins were treated with H-89. The aim of this experiment was to demonstrate that the FRET-based kinase assay is a robust technique and is inherently competent to exclude false positives. H-89 is a specific PKA inhibitor (Marunaka et al., 2003) and it should not have any effect on the kinase activity of the purified GSK-3β protein. As observed in (Fig. 2c) H-89 treatment has no effect on the kinase activity of GSK-3β, whereas it leads to a decrease in the activity of PKA enzyme only**.**

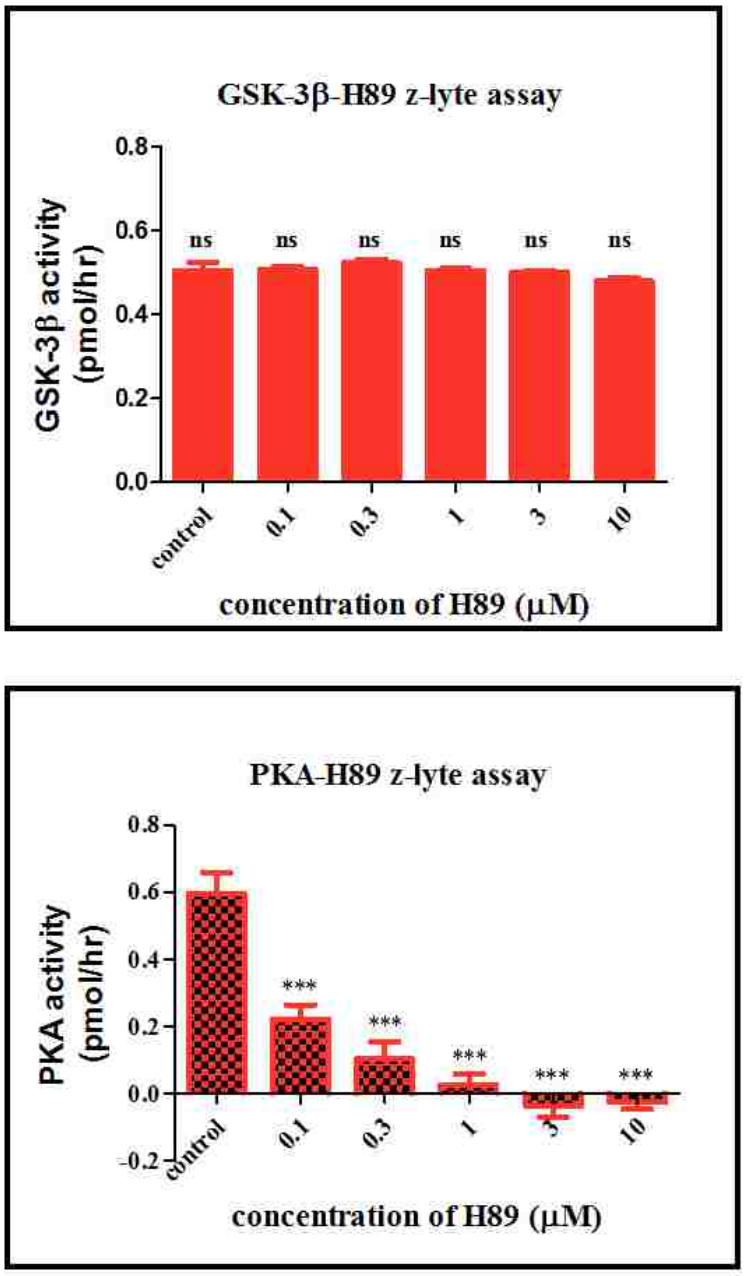


Fig 1. H-89 inhibits the in vitro kinase activity of PKA but not GSK-3β. Pure recombinant GSK-3β (*filled bars*) and PKA (*pattern bars*) proteins were used in the FRET-based kinase assay and the enzyme activity was measured in the absence (control) or in the presence of 0.1, 0.3, 1, 3 or 10 μM H-89. Activity is expressed as the rate of formation of phosphorylated peptide substrate (mean +/- SD). Data was analyzed using one-way ANOVA ($P < 0.05$ considered significant compared to control) by Tukey's multiple comparison test. GraphPad Prism version 5.0 for windows, GraphPad Software, La Jolla California USA, www.graphpad.com was used. (ns = non significant compared to control, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$).

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