รายงานวิจัยฉบับสมบูรณ์

ทุนพัฒนานักวิจัย
สัญญาเลขที่ RSA5180008

กลไกระดับโมเลกุลของการเพิ่มการตายแบบเอพ็อพโตสิสโดย ไกลโคเจน สินเทส ไคเนส-3: การทำงานผ่านทางไมโตคอนเดรีย p53 และ VEGF

Molecular mechanism of Glycogen Synthase Kinase 3 (GSK3) in promoting apoptosis: action on mitochondria p53, and VEGF

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บทคัดย่อ

การเกิดการตายแบบอะพ็อพโทสิส (apoptosis) มีความสำคัญต่อหน้าที่ของเซลล์ทั้งในสภาวะปกติและภาวะที่เป็นโรคต่างๆอาทิ มะเร็ง เบาหวาน และโรคที่มีภาวะการเสื่อมสภาพของเซลล์ประสาท Glycogen synthase kinase-3 (GSK3) และ p53 มีบทบาทสำคัญในการเกิดการตายแบบอะพ็อพโทสิสที่เกี่ยวข้องกับไมโตคอนเดรีย มีรายงานว่า p53 จำเป็นสำหรับการเกิดการตายแบบอะพ็อพโทสิสในเมื่อมี overexpression ของ GSK3 ผลของโครงการวิจัยนี้แสดงให้เห็นว่า GSK3 มีส่วนเสริมการเพิ่มขึ้นของ p53 และการตายแบบอะพ็อพโทสิสที่กระตุ้นโดย DNA damage อีกทั้งยังส่งเสริมการเคลื่อนย้ายของ p53 ไปสู่ไมโตคอนเดรียและส่งเสริมให้ cytochrome C หลุดออกจากไมโตคอนเดรียมาสู่ cytosol ซึ่งเป็นลักษณะเฉพาะของการตายแบบอะพ็อพโทสิสที่ผ่านทางไมโตคอนเดรีย อย่างไรก็ตามการส่งเสริมการเคลื่อนย้ายของ p53 ไปสู่ไมโตคอนเดรียโดย GSK3 นั้นเกิดกับ wild-type p53 เท่านั้นไม่เกิดกับ mutant p53 การจับกันของ p53 กับ Bcl2 ที่ไมโตคอนเดรียและทำให้เกิด Bax oligomerization ที่กระตุ้นโดย DNA damage นั้นพบว่าลดลงเมื่อการทำงานของ GSK3 ถูกยับยั้ง นอกจากนี้ยังพบว่า overexpression ของ GSK3 ทั้งแบบ wild-type, constitutively active และ kinase dead กระตุ้นการเพิ่มปริมาณของ p53 ในสภาวะ unstressed โดยผ่านทางการเพิ่ม p53 stability siRNA GSK3 ทำให้ p53 ในเซลล์ลดลง แสดงว่า GSK3 มีส่วนในทำให้ปริมาณของ p53 ในภาวะปกติคงตัว เป็นที่ทราบกันดีอยู่แล้วว่าการยับยั้งการทำงานของ GSK3 สามารถป้องกันการตายแบบอะพ็อพโทสิสได้ซึ่งสาเหตุอันหนึ่งอาจมาจากการที่ GSK3 สามารถยับยั้ง transcription ของ VEGF ดังนั้นผู้วิจัยจึงศึกษาความเชื่อมโยงของการป้องกันการตายแบบอะพ็อพโทสิสโดยการยับยั้งการทำงานของ GSK3 และ VEGF expression ผลการศึกษาพบว่าการยับยั้ง VEGF โดยใช้ SU4312 ไม่สามารถระงับผลของการยับยั้ง GSK3 ที่ทำให้เกิดการลดลงของการตายแบบอะพ็อพโทสิสได้ ซึ่งชี้ให้เห็นว่าผลการป้องกันการตายแบบอะพ็อพโทสิสที่เกิดจากการยับยั้งการทำงานของ GSK3 นั้นไม่เกี่ยวข้องกับการยับยั้ง transcription ของ VEGF โดย GSK3

โดยสรุปจากโครงการวิจัยพบว่า 1) GSK3 มีส่วนเสริมให้เกิดการเคลื่อนย้ายของ p53 ไปสู่ไมโตคอนเดรีย ที่ซึ่ง p53 จับกับ Bcl2 และทำให้เกิด Bax oligomerization และ cytochrome C release นำไปสู่การกระตุ้นการทำงานของ caspase และเกิดการตายแบบอะพ็อพโทสิสที่ผ่านทางไมโตคอนเดรีย 2) การเพิ่มปริมาณของ GSK3 ทำให้ปริมาณของ p53 เพิ่มขึ้นในสภาวะ unstressed โดยผ่านทางการเพิ่ม p53 stability การนี้ ไม่ขึ้นกับ kinase activity ของ GSK3 3) การป้องกันการตายแบบอะพ็อพโทสิสที่เกิดจากการยับยั้งการทำงานของ GSK3 นั้นไม่เกี่ยวข้องกับการที่ GSK3 สามารถการยับยั้ง transcription ของ VEGF

Abstract

Apoptosis is very important in normal cellular functions and pathology of many diseases including cancer, diabetes and neurodegeneration. Most of cell death in vertebrates occurs through mitochondria signaling pathway of apoptosis. Glycogen synthase kinase 3 (GSK3) and p53 play crucial roles in the mitochondrial apoptotic pathway, and ectopic expression of GSK3 has been shown to induce apoptosis through p53 dependent manner. The present study demonstrated that GSK3 promoted DNA damage-induced p53 accumulation and apoptosis as pharmacological inhibitor of GSK3 attenuated p53 accumulation and apoptosis induced by DNA damage. Furthermore, DNA damage induced translocation of p53 to the mitochondria and the release of cytochrome c from mitochondria, and both of these apoptotic responses were attenuated by inhibition of GSK3 suggesting that GSK3 promotional action on DNA damage-induced p53 mitochondrial translocation and mitochondrial apoptosis pathway. However, regulation of p53 mitochondrial translocation by GSK3 was only evident with wild-type, not mutated, p53. Additionally, inhibition of GSK3 reduced etoposide-induced association of p53 with Bcl2 and Bax oligomerization. As our finding indicates that GSK promotes p53 accumulation upon DNA damage stress, we examined whether GSK3 influenced p53 in unstressed condition. Overexpression of wild-type, constitutively active S9A, and kinase dead (KD) GSK3β caused increase in level of basal p53 without affecting p53 phosphorylation at GSK3 site, serine-33. These indicate that GSK3 is capable inducing p53 independent of its kinase activity. GSK3β induced increase in basal p53 by increase p53 stability as co-expression of GSK3β S9A and p53 caused delay in degradation of p53 when protein synthesis was inhibited by cyclohexamide. GSK3 knock down by siRNA caused reduction of basal p53 confirming that GSK3 contributed to maintaining of p53 level at basal condition. The apoptotic protective of GSK3 inhibition is well documented, and a possible link to this action is that GSK3 suppresses transcription of VEGF gene. Therefore, we investigated whether antiapoptotic action of GSK3 inhibition was involved increase prosurvival signal by VEGF induction. The results showed that inhibition of GSK3 increased VEGF expression however VEGF may not play a role in protective action of GSK3 inhibition as inhibition of VEGF signaling by inhibiting VEGF receptor 2 kinase using its inhibitor, SU4312 could not reverse GSK3 inhibition protection against camptothecin-induced apoptosis.

Collectively, the present study reveals that 1) GSK3 promotes the mitochondrial translocation of wild-type but not mutated p53, where p53 interacts with Bcl2 to allow Bax oligomerization and the subsequent release of cytochrome C that leads to caspase activation in the mitochondrial pathway of intrinsic apoptotic signaling, 2) increase in GSK3 level augments p53 in basal condition through mechanism involves increase its stability, 3) the antiapoptotic action of GSK3 inhibition may not involve its ability to suppress VEGF expression.

หน้าสรุปโครงการ (Executive Summary)
ทุนพัฒนานักวิจัย

ชื่อโครงการ (ภาษาไทย) **กลไกระดับโมเลกุลของการเพิ่มการตายแบบเอพ็อพโตสิสโดยไกลโคเจน สินเทส ไคเนส-3: การทำงานผ่านทางไมโตคอนเดรีย p53 และ VEGF**

(ภาษาอังกฤษ) **Molecular mechanism of Glycogen Synthase Kinase 3 (GSK3) in promoting apoptosis: action on mitochondria p53, and VEGF**

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1. ความสำคัญและที่มาของปัญหา

Apoptosis is very important in normal cell functions and pathology of numerous diseases including cancer, and neurodegenerative diseases such as Alzheimer and Parkinson. Most of cell death in vertebrates occurs through mitochondria signaling pathway of apoptosis. Hence, regulation of mitochondria pathway of apoptosis becomes an important therapeutic target for many diseases. In deed, pharmacological modulators of apoptosis are in clinical trial and research for therapeutics.

The tumor suppressor protein, p53 is important in cell survival and cancer development so it is important to find mechanisms that regulate p53 that might provides therapeutic targets for controlling p53 actions. Although p53 can induce apoptosis through its transcription activity in regulating expression of proapoptotic and antiapoptotic proteins, its transcriptional independent action in apoptosis is widely established. Recently, it has been disclosed that p53 mitochondria translocation is the key to this action of p53.

Glycogen synthase kinase-3 (GSK3) is a remarkable enzyme. It phosphorylates over 30 known substrates, including the tumor suppressor protein, p53. Inadequately controlled GSK3β activity has been implicated in number of diseases, including Alzheimer's disease, diabetes and cancer. Additionally, GSK3 has been reported to promote apoptosis induced by a variety of

apoptotic stimuli. Recently, we demonstrated for the first time that GSK3 facilitates p53 mitochondrial translocation following arsenic-mediated apoptosis. Therefore, it is of interest to elucidate the mechanism of p53 mitochondrial translocation regulated by GSK3, and to explore new role for GSK3 in apoptosis.

The overall goal is to investigate mechanism by which GSK3 controlling p53 mitochondrial translocation and apoptosis, and mechanisms regulating GSK3-mediated apoptosis. The anticipated outcomes of this research project will further our understanding in regulation of p53-mediated mitochondria signaling pathway of apoptosis by GSK3 which subsequently will provide a new target for therapeutics of diseases whose pathology are associated with apoptosis.

2. วัตถุประสงค์

Specific Aim 1: will test the hypothesis that p53 mitochondrial translocation is regulated by GSK3.

Specific Aim 2: will test the hypothesis that GSK3 promotes the induction of mitochondria signaling pathway of apoptosis.

Specific Aim 3: will test the hypothesis that GSK3 promotes apoptosis through its modification of VEGF expression.

3. สรุปผลการวิจัย

Collectively, the present study reveals that 1) GSK3 promotes the mitochondrial translocation of wild-type but not mutated p53, where p53 interacts with Bcl2 to allow Bax oligomerization and the subsequent release of cytochrome C that leads to caspase activation in the mitochondrial pathway of intrinsic apoptotic signaling, 2) increase in GSK3 level augments p53 in basal condition through mechanism involves increase its stability, 3) the antiapoptotic action of GSK3 inhibition may not involve its ability to suppress VEGF expression.

4. ผลงานที่ได้จากงานวิจัย

-Poster presentation: The 3rd Biochemistry and Molecular Biology (BMB) Conference "From Basic to Translational Research for a Better Life", April 6-8, 2011, Chaingmai, Thailand

-Manuscript: Patchara Ngok-ngam, Piyajit Watcharasit, Apinya Thiantanawat, and Jutamaad Satayavivad. Pharmacologic modulation of GSK3 attenuated DNA damage-induced apoptosis via regulation of p53 mitochondrial translocation and Bax oligomerization in neuroblastoma SH-SY5Y cells. (In preparation) (ภาคผนวก 1)

5. ประโยชน์ที่ได้รับจากโครงการวิจัย

-เผยแพร่ผลงานวิจัยในการประชุมวิชาการระดับนานาชาติ (The 3rd Biochemistry and Molecular Biology (BMB) International Conference "From Basic to Translational Research for a Better Life", April 6-8, 2011, Chaingmai, Thailand)

-คาดว่าผลงานวิจัยนี้น่าจะสามารถตีพิมพ์ในวารสารวิชาการระดับนานาชาติได้ ขณะนี้กำลังเตรียม Manuscript เพื่อจะส่งไปตีพิมพ์

รายงานโครงการวิจัยฉบับสมบูรณ์

ชื่อโครงการ (ภาษาไทย) **กลไกระดับโมเลกุลของการเพิ่มการตายแบบเอพ็อพโตสิสโดยไกลโคเจน สินเทส ไคเนส-3: การทำงานผ่านทางไมโตคอนเดรีย p53 และ VEGF**

(ภาษาอังกฤษ) **Molecular mechanism of Glycogen Synthase Kinase 3 (GSK3) in promoting apoptosis: action on mitochondria p53, and VEGF**

สัญญาเลขที่ RSA5180008

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1. ความสำคัญและที่มาของปัญหา

Background and rationale

Apoptosis is very important in normal cell functions and pathology of numerous diseases including cancer, and neurodegenerative diseases such as Alzheimer and Parkinson. Most of cell death in vertebrates occurs through mitochondria signaling pathway of apoptosis. Hence, regulation of mitochondria pathway of apoptosis becomes an important therapeutic target for many diseases. In deed, pharmacological modulators of apoptosis are in clinical trial and research for therapeutics.

The tumor suppressor protein, p53 is important in cell survival and cancer development so it is important to find mechanisms that regulate p53 that might provides therapeutic targets for controlling p53 actions. Although p53 can induce apoptosis through its transcription activity in regulating expression of proapoptotic and antiapoptotic proteins, its transcriptional independent action in apoptosis is widely established. Recently, it has been disclosed that p53 mitochondria translocation is the key to this action of p53.

Glycogen synthase kinase-3 (GSK3) is a remarkable enzyme. It phosphorylates over 30 known substrates, including the tumor suppressor protein, p53. Inadequately controlled GSK3β activity has been implicated in number of diseases, including Alzheimer's disease, diabetes and cancer. Additionally, GSK3 has been reported to promote apoptosis induced by a variety of apoptotic stimuli. Recently, we demonstrated for the first time that GSK3 facilitates p53 mitochondrial translocation following arsenic-mediated apoptosis. Therefore, it is of interest to elucidate the mechanism of p53 mitochondrial translocation regulated by GSK3, and to explore new role for GSK3 in apoptosis.

The overall goal is to investigate mechanism by which GSK3 controlling p53 mitochondrial translocation and apoptosis, and mechanisms regulating GSK3-mediated apoptosis. The anticipated outcomes of this research project will further our understanding in regulation of p53-mediated mitochondria signaling pathway of apoptosis by GSK3 which

subsequently will provide a new target for therapeutics of diseases whose pathology are associated with apoptosis.

2. วัตถุประสงค์

Specific Aims

Specific Aim 1: will test the hypothesis that p53 mitochondrial translocation is regulated by GSK3.

Specific Aim 2: will test the hypothesis that GSK3 promotes the induction of mitochondria signaling pathway of apoptosis**.**

Specific Aim 3: will test the hypothesis that GSK3 promotes apoptosis through its modification of VEGF expression

3. ผลการวิจัย

Results

GSK3 inhibition attenuated DNA damage-induced p53 accumulation and apoptosis

To study p53-mediated apoptosis signaling, we used three mechanistically different DNA damaging agents that induce p53 expression: doxorubicin, a DNA intercalating agent, etoposide, a topoisomerase II inhibitor, and camptothecin, a topoisomerase I inhibitor. Treatment of human neuroblastoma SH-SY5Y cells, which express wild-type p53, with doxorubicin, etoposide, or camptothecin caused large increases in level of p53 (Fig. 1). Activation of apoptosis was evident by increases in the cleavage of PARP, which is a substrate of caspase 3, a key enzyme activated during apoptosis (Fig. 1). Levels of the anti-apoptotic protein, Bcl2, and the pro-apoptotic protein, Bax, were not affected by doxorubicin, etoposide or camptothecin treatments (Fig. 1), suggesting that apoptosis induced by the three inducers of DNA damage was not due to imbalances of these proteins.

To test whether GSK3 is involved in the regulation of p53 induction and apoptosis, two mechanistically different selective inhibitors of GSK3, lithium and SB216763, were used. GSK3 activity is mainly regulated by phosphorylation, with activity inhibited by N-terminal serine-phosphorylation (serine-21 for GSK3α and serine-9 for GSK3β), and activity increased by tyrosine phosphorylation (tyrosine-279 for GSK3α and tyrosine-216 for GSK3β). GSK3 inhibition by lithium caused increases in the serine phosphorylation of both GSK3 isoforms, as reported previously. SB216763 directly inhibits GSK3 by competition at the ATP binding site, which resulted in decreases of both serine and tyrosine-phosphorylation of GSK3α/β (Fig. 1). Pretreatment with lithium or SB216763 reduced the induction p53 and cleavage of PARP induced by treatment with doxorubicin, etoposide and camptothecin (Fig. 1), indicating that

GSK3 contributes to the induced expression of p53 and apoptosis caused by each of these DNA damaging agents. However, lithium and SB216763 did not alter the levels of Bcl2 or Bax (Fig. 1), indicating that the apoptotic protection by GSK3 inhibition is unlikely to be through modulation of the levels of Bcl2 or Bax.

Inhibition of GSK3 reduces p53 translocation to mitochondria and apoptosis

Since translocation of p53 to the mitochondria is capable of inducing apoptosis, we investigated the contribution of GSK3 to p53 mitochondrial translocation and its activation of mitochondria-mediated apoptosis. SH-SY5Y cells were treated with doxorubicin (2 μM), etoposide (10 μM) or camptothecin (5 μM) for 5 hr, and mitochondrial, cytosolic, and nuclear fractions were prepared. The purity of the mitochondrial, cytosolic and nuclear preparation was verified by immunoblotting for marker of each fraction, ATP synthase F1 subunit, β-tubulin and histone H1, respectively (Fig. 2A). Treatment with doxorubicin, etoposide, or camptothecin caused significant increases in the levels of p53 in the mitochondria (Fig. 2B), and cytochrome C release into the cytosol (Fig. 2C), which indicates activation of the mitochondrial apoptosis pathway. Inhibition of GSK3 by lithium or SB216763 prominently attenuated p53 translocation to the mitochondria following doxorubicin, etoposide and camptothecin treatment (Fig. 2B). Quantitative analysis revealed that inhibition of GSK3 with lithium or SB216763 significantly reduced DNA damage-induced p53 translocation to mitochondria (Fig. 2C) while it did not alter the levels of p53 in the cytosol and nucleus (Fig. 2B). The results indicate that active GSK3 contributes to p53 mitochondrial translocation upon apoptosis. Additionally, GSK3 inhibition reduced cytochrome C release (Fig. 3). These results indicate that GSK3 promotes both p53 mitochondrial translocation and the mitochondrial apoptosis pathway.

GSK3 regulates mitochondrial translocation of wild-type but not mutant p53

It has been reported that some transcriptionally incompetent mutants of p53 are capable of inducing apoptosis and are present in the mitochondria after induction of apoptosis. Therefore, we investigated whether GSK3 controls mitochondrial translocation of mutant p53. To do this, breast cancer T47D cells expressing L194F mutant p53 was used. Unlike wild-type p53, which is expressed at very low levels in unstressed cells, mutant p53 is expressed at high levels in untreated T47D cells (Fig. 4A). Additionally, we found that higher levels of mutant p53 than wild-type p53 is in the mitochondria of untreated cells (Fig. 4A). Furthermore, inhibition of GSK3 by lithium did not affect the basal levels of mutant p53 in the mitochondria in T47D cells (Fig. 4B). Moreover, treatment with etoposide did not significantly affect the mitochondrial levels of mutant p53, and GSK3 inhibition did not alter mitochondrial mutant p53 levels following treatment with etoposide (Fig. 4C). These results indicate that GSK3 only regulates the mitochondrial translocation of wild-type p53, but not mutant p53, or that GSK3 only regulates

the increase in mitochondrial p53 that follows activation of apoptotic signaling, which was absent in T47D cells.

GSK3 inhibition only affects phosphorylation of serine 33 of p53 induced by DNA damage

It has been shown that p53 can be phosphorylated by multiple kinases including GSK3 (at serine-33, 315 and 376) in response to a variety of stresses, and phosphorylation of p53 has been shown to influence its cellular localization. Thus, alteration of p53 phosphorylation may contribute to attenuation of p53 mitochondrial translocation by GSK3 inhibition. To test this hypothesis, we first examined whether p53 post-translation modification was affected by GSK3 inhibition. SH-SY5Y cells were treated with etoposide with or without pretreatment of lithium to inhibit GSK3 activity. The mitochondrial lysates were subjected to 2-D gel electrophoresis, and p53 was detected by immunoblot. The results showed that there was a decrease p53 immunoreactivity suggesting reduction of post-translation modification of p53 by GSK3 inhibition (Fig. 5). We further examined whether GSK3 inhibition affected phosphorylation modification of p53 using phospho-specific p53 antibodies. The results showed that treatment with DNA damaging agents, camptothecin, etoposide and doxorubicin caused increase in levels of mitochondrial p53 phosphorylation at serine-15, 33, and 37. Inhibition of GSK3 by lithium reduced DNA damage-induced phospho-serine-33 p53 in the mitochondria without altering p53 phosphorylation at serine-37, which is a phosphorylation primed site for GSK3, and at serine-15 suggesting that inhibition of GSK3 did not alter activation of other kinases by DNA damage, and that at least in part GSK3 involved in DNA damage-induced p53 phosphoryaltion at serine-33 (Fig. 6). However, the conclusion regarding contribution of serine-33 phosphorylation could not be made as the reduction in p53 phosphorylation at serine-33 was also observed in the nucleus and cytosol.

Inhibition of GSK3 reduces the interaction of p53 with Bcl2 and Bax oligomerization

It has been shown that p53 directly induces mitochondrial apoptosis through its interaction with Bcl2, a key protein regulating the mitochondrial outer membrane permeability. Thus, we examined the p53-Bcl2 interaction in mitochondria by co-immunoprecipitation. The level of p53 in mitochondria was increased, whereas level of Bcl2 was unaltered, by etoposide treatment (Fig. 7A). Co-immunoprecipitation of p53 and Bcl2 was observed in etoposide-treated cells, and this p53-Bcl2 interaction was drastically reduced by treatment with the GSK3 inhibitor lithium (Fig. 5A). We further investigated whether GSK3 inhibition affected Bax oligomerization, which can lead to mitochondrial outer membrane permeabilization and subsequently cytochrome C release. To detect Bax oligomerization, after treatments mitochondrial proteins were cross-linked by treatment with EGS, followed by western blot analysis. The results show that etoposide treatment increased immunoreactivity of a slow migrating band above the 21 kD

Bax monomer band (Fig. 7B) indicating increased Bax oligomerization associated with the mitochondria following etoposide. Inhibition of GSK3 with lithium treatment attenuated etoposide-induced Bax oligomerization in the mitochondria. The level of Bax in the mitochondria was not altered by either etoposide or lithium thus the reduction of Bax oligomerization by GSK3 inhibition was not due to decrease of Bax in the mitochondria.

p53-β-catenin interaction in mitochondria

We observed p53-β-catenin interaction in the mitochondria by co-immunoprecipitation technique, and etoposide increased interaction between p53 and β-catenin and this interaction was reduced by inhibition of GSK3 (figure 8). Although, coimmunoprecipitation of p53 and β-catenin was also observed in the cytosol, the interaction seemed to be opposite to what observed in the mitochondria as etoposide reduced p53-β-catenin interaction in the cytosol and the interaction was returned to of control level when GSK3 was inhibited (figure 8).

Overexpression of GSK3 causes p53 accumulation

Our finding indicates that GSK promotes p53 accumulation upon DNA damage led us to explore whether GSK3 influenced p53 in unstressed condition. To test the effect of GSK3 on basal level of p53, wild-type GSK3β, constitutively active S9A mutant GSK3β (GSK3β-S9A), and kinase dead GSK3β (GSK3β-KD) were overexpressed in HEK293 cells which endogenously express wild-type p53 and p53 level was determined by immunoblot analysis. Overexpression of all 3 constructs of GSK3β increased basal levels of p53 in HEK293 cells (Fig. 9A). Quantitative analysis of p53 immunoreactivity bands demonstrated that the increase of p53 levels by overexpression of GSK3β was statistically significant from vector control (Fig. 9B). The finding that increase in basal level of p53 by overexpression of wild-type GSK3β, constitutively active GSK3β-S9A, and kinase dead GSK3β suggests that only level but not its activity of GSK3 is necessary for GSK3 mediated p53 induction in basal unstressed condition. This notion was in agreement with the result that overexpression of wild-type or constitutively active GSK3β-S9A did not affect level of p53 phosphorylation at serine-33 which is a GSK3 site (Fig. 9B). We further tested that whether level of GSK3 present in cell was contributed to maintaining basal level of p53, GSK3 level was knockdown by siRNA GSK3α/β. SiRNA GSK3α/β caused about 40-50% reduction in GSK3 level (Fig. 10), and this reduction caused small but significant decrease in basal level of p53 (Fig. 10). This suggests the role of GSK3 in maintaining p53 stability in unstressed condition.

Overexpression of GSK3β increase p53 stability

To investigate how GSK3β caused increase in p53 level, we tested whether GSK3β overexpression caused increase in p53 by changing its stability. Since p53 expression is very low in basal, we used exogenous p53 expression to overcome this problem. Wild-type p53 was co-transfected with vector or GSK3β-S9A in H1299 p53 null cells. 24 hours after transfection

cyclohexamide (20 μg/ml) was added to inhibit protein synthesis, and loss of p53 expression over time was monitored. The result showed that the loss of p53 expression was delayed by overexpression of GSK3β-S9A (Fig. 11) indicating that stability of p53 is increased by GSK3β overexpression. Additionally, GSK3-mediated p53 phosphorylation was probably not be responsible for its induction of p53 stabilization as overexpression of GSK3 did not increase p53 phosphorylation at GSK3 site, serine-33.

Inhibition of GSK3 caused VEGF expression

Inhibition of GSK3 has been shown to have protective effect against a variety of apoptotic stimuli. One of possible mechanisms by which GSK3 inhibition provides protection against apoptosis has been recently reported that is inhibition of GSK3 induces VEGF, a key growth factor participating in promotion of growth and survival. Therefore, we aimed to examine that whether inhibition of GSK3 provides protection from apoptosis through its modification of VEGF expression. We first sought out the condition which GSK3 inhibition induced VEGF expression in neuroblastoma SH-SY5Y and human microendthelial (HMEC) cells. Using ELISA technique to detect VEGF protein secreted in the medium, we found that treatment of 20 mM lithium or 20 μM SB216763 increased levels of VEGF secreted in the medium when compared to vehicle control (Fig. 12). These results ensured us that inhibition of GSK3 leads to increase in expression of VEGF. We further examined that whether inhibition of GSK3 provides protection from apoptosis through its modification of VEGF expression by inhibiting VEGF signaling using VEGF receptor 2 kinase inhibitor, SU4312. Inhibition of GSK3 protected both SH-SY5Y and HMEC cells from camptothecin-induced apoptosis as lithium reduced caspase 3 activation by camptothecin in both cell lines (Fig. 14). However, co-treatment of SU4312 and lithium did not reverse the protective action of lithium against camptothecin-induced caspase-3 activation (Fig. 13). These results suggested that the apoptotic protective action of GSK3 inhibition may not be due to its ability to induce VEGF expression.

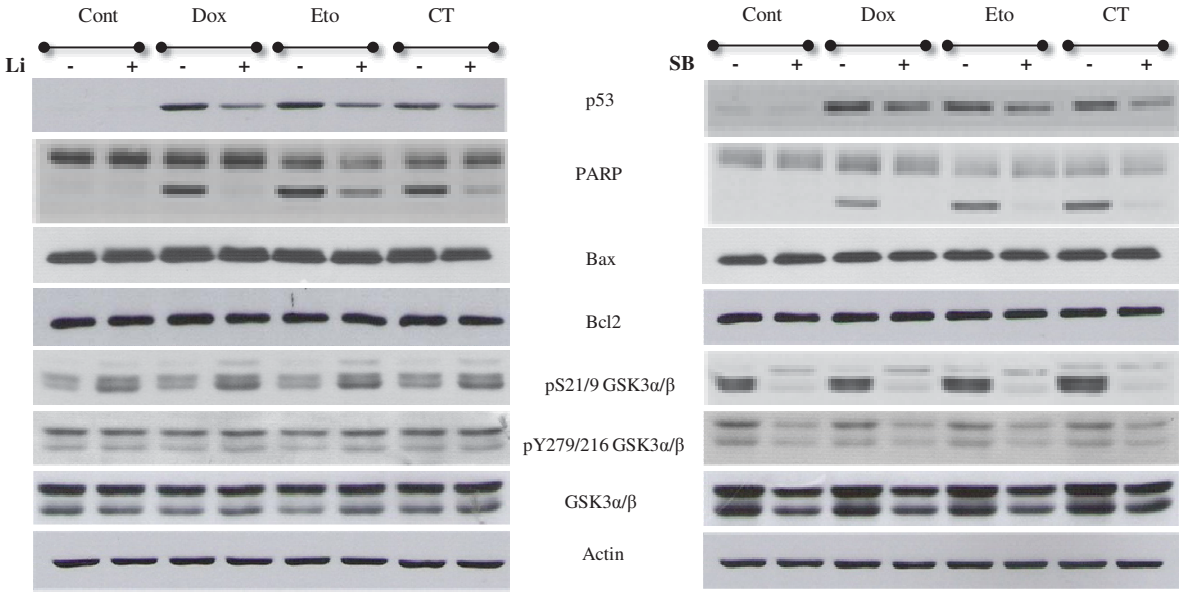
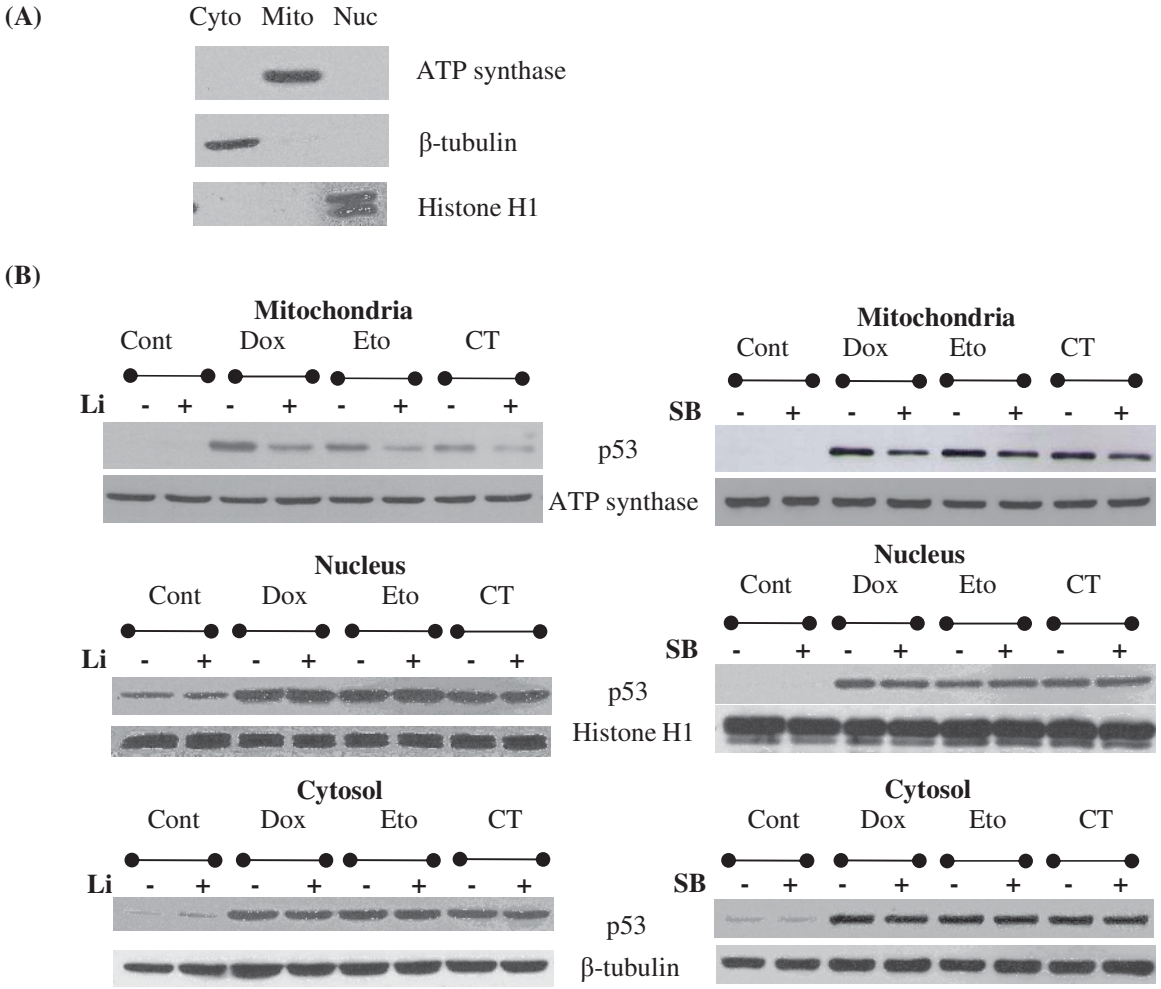


Figure 1. GSK3 inhibition attenuates DNA damage-induced increased p53 levels and caspase activation SH-SY5Y cells were treated with doxorubicin (Dox, 2 μM), etoposide (10 μM, Eto), or camptothecin (CT, 5 μM) for 5 hr with or without a 30 min pretreatment with GSK3 inhibitors, lithium (Li, 20 mM) or SB216763 (SB, 20 μM). Levels of p53, PARP, pS21/9-GSK3α/β, pY279/216-GSK3α/β, GSK3α/β, Bcl2, and Bax were analyzed by immunoblot analysis and β-actin was used as a loading control. Caspase activation was determined by measuring PARP proteolysis.


(A)
Cyto Mito Nuc
ATP synthase
β-tubulin
Histone H1
(B)
Mitochondria
Cont Dox Eto CT
Li - + - + - + - +
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ATP synthase
Mitochondria
Cont Dox Eto CT
SB - + - + - + - +
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ATP synthase
Nucleus
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p53
β-tubulin
Cytosol
Cont Dox Eto CT
SB - + - + - + - +
p53
β-tubulin
(C)
Mitochondrial p53
without inhibitor
+ Li
+ SB
p53 (% without inhibitor)
120
100
80
60
40
20
0
*
Dox
Eto
CT

Figure 2. Inhibition of GSK3 reduced p53 mitochondrial translocation

The mitochondrial, nuclear, and cytosolic fractions were prepared from cells treated with doxorubicin, etoposide, or camptothecin for 5 hr with or without a 30 min pretreatment with GSK3 inhibitors, lithium or SB216763. (A) Mitochondria (Mito), cytosolic (Cyto), and nuclear (Nuc) fractions were immunoblotted with a protein marker for each fraction, the F1 subunit ATP synthase for mitochondria, β-tubulin for the cytosol, and histone H1 for the nucleus to verify the purity of each fraction. (B) The levels of mitochondrial, cytosolic and nuclear p53 were detected by immunoblotting. (C) The quantitative data of mitochondrial p53 were expressed as % individual chemical treatment without GSK3 inhibition (mean $\pm$ SD; n=3), * $p < 0.05$.

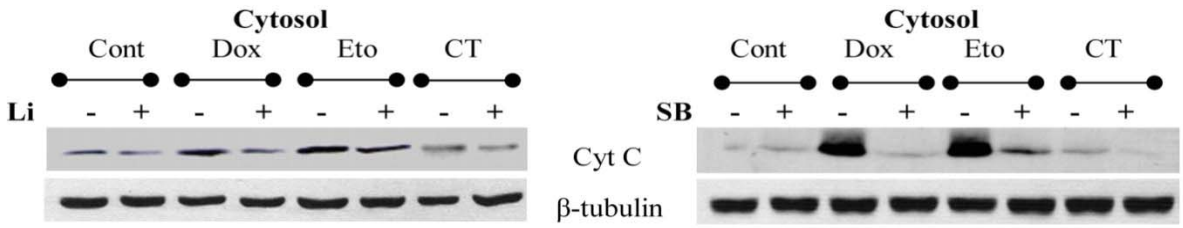

Figure 3. Inhibition of GSK3 reduced cytochrome c release from the mitochondria

SH-SY5Y cells were treated with doxorubicin (Dox, 2 μM), etoposide (10 μM, Eto), or camptothecin (CT, 5 μM) for 5 hr with or without a 30 min pretreatment with GSK3 inhibitors, lithium (Li, 20 mM) or SB216763 (SB, 20 μM), and cytochrome C released into the cytosol was measured by immunoblotting.

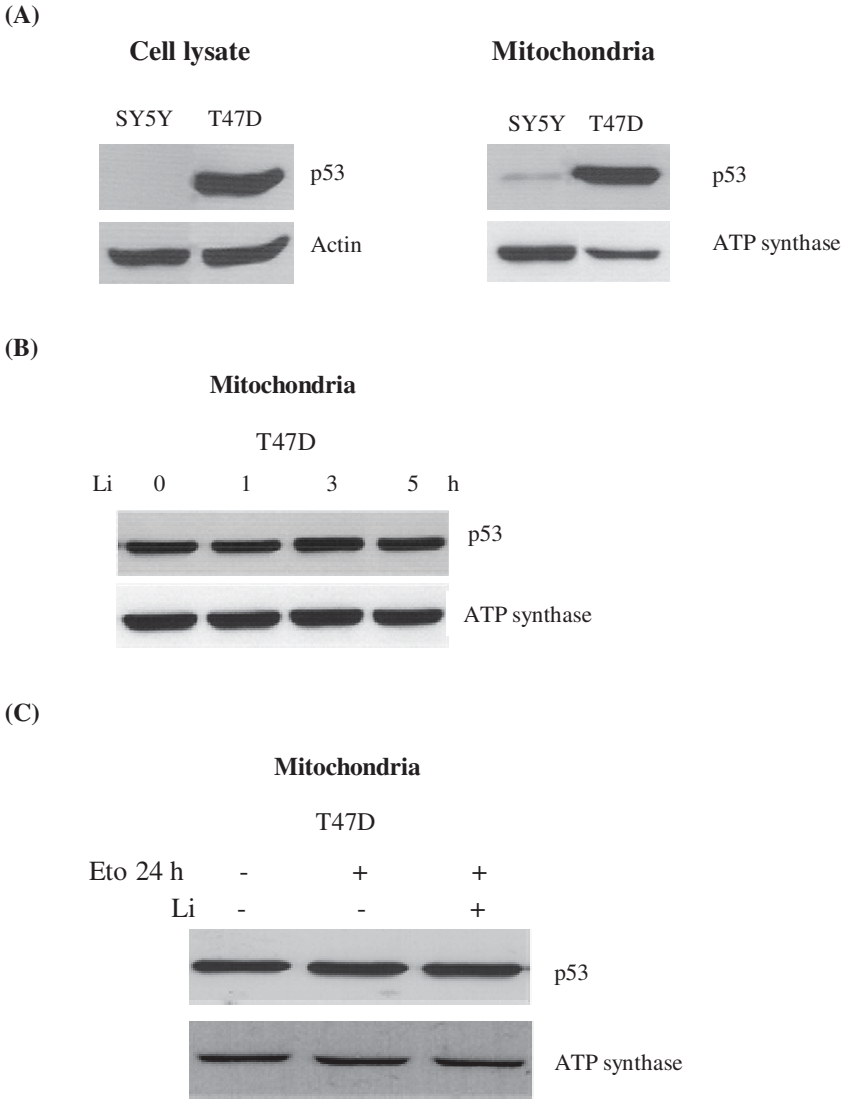

Figure 4. GSK3 regulates the mitochondrial translocation of wild-type but not mutant p53
(A) Levels of mitochondrial p53 in SH-SY5Y (wild-type p53) and T47D (L194F p53) cells. (B) T47D cells were treated with 20 mM lithium for 1, 3 or 5 hr and the levels of mitochondrial p53 were measured by immunoblot analysis. (C) T47D cells were treated with etoposide (10 μM) with or without 20 mM lithium for 24 hr and the levels of mitochondrial p53 were analyzed by immunoblotting.

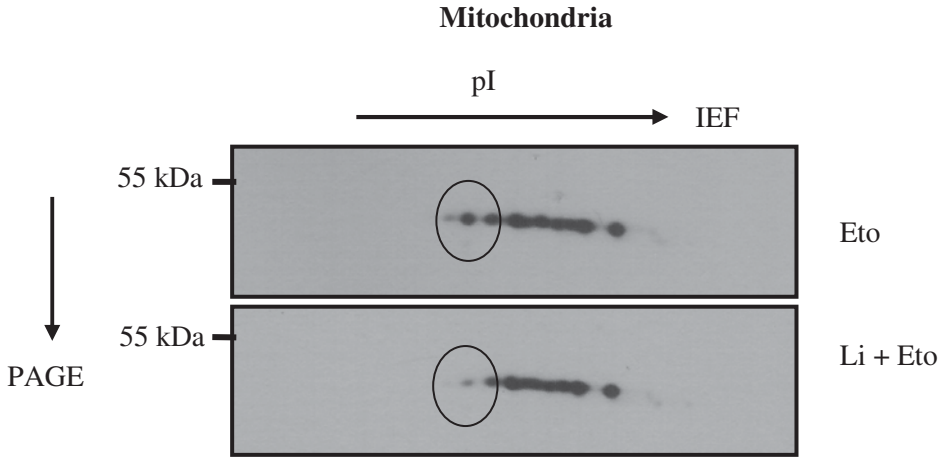

Figure 5. Immunoblot analysis of p53 by 2D-PAGE

Mitochondrial lysates from SH-SY5Y cells treated with etoposide with or without lithium pretreatment were subjected to 2D-PAGE, transferred onto PVDF membranes and immunoblotted with p53 antibody. The p53 immunoreactivity was visualized using enhanced chemiluminescence. The different in density of p53 immunoreactivity in etoposide treated with and without lithium was highlighted in circles.

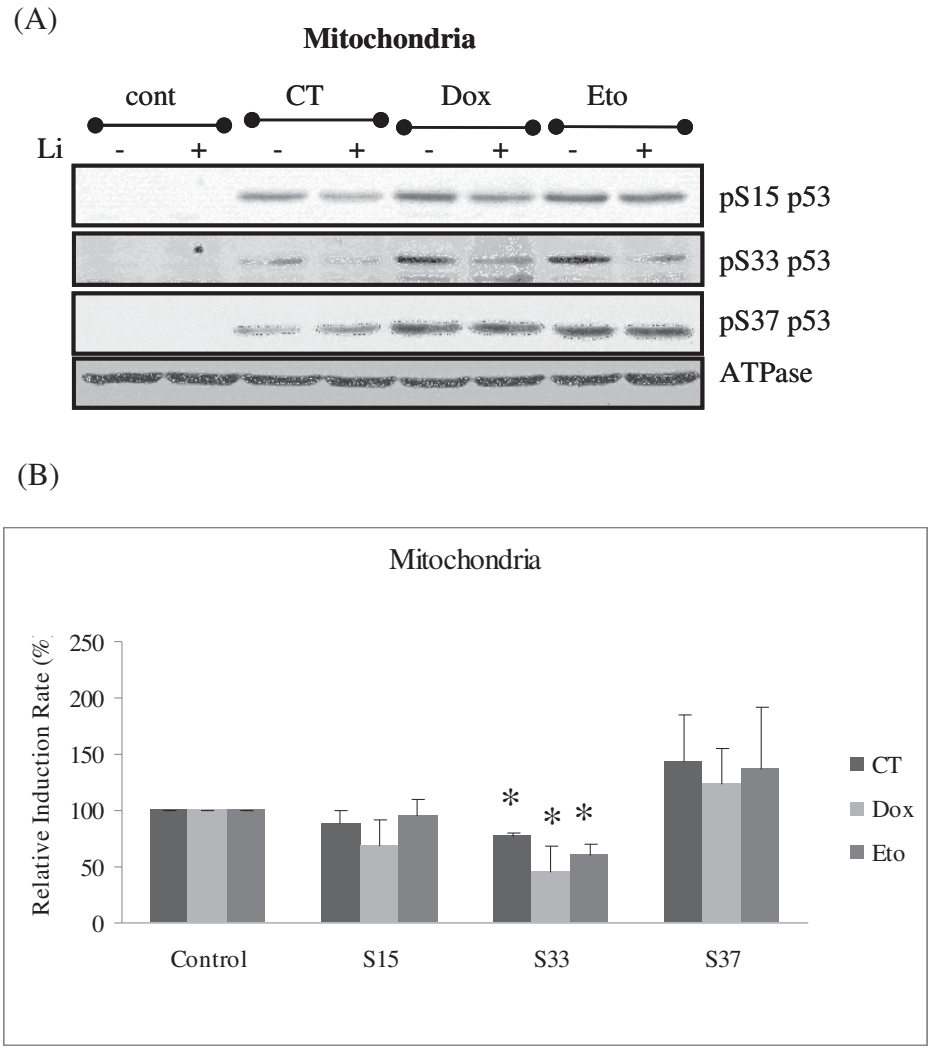

Figure 6. GSK3 inhibition only affects phosphorylation of serine 33 of p53 induced by DNA damage

SH-SY5Y cells were treated with camptothecin, doxorubicin or etoposide with or without pretreatment of lithium. Phosphorylation of serine-15, 33, and 37 p53 in the mitochondria were detected by immunoblot analysis using phosphospecific antibodies (A), and the results were quantified and presented as mean ± SE of % of camptothecin, camptothecin, doxorubicin or etoposide alone. * represented statistically different at $p<0.05$.

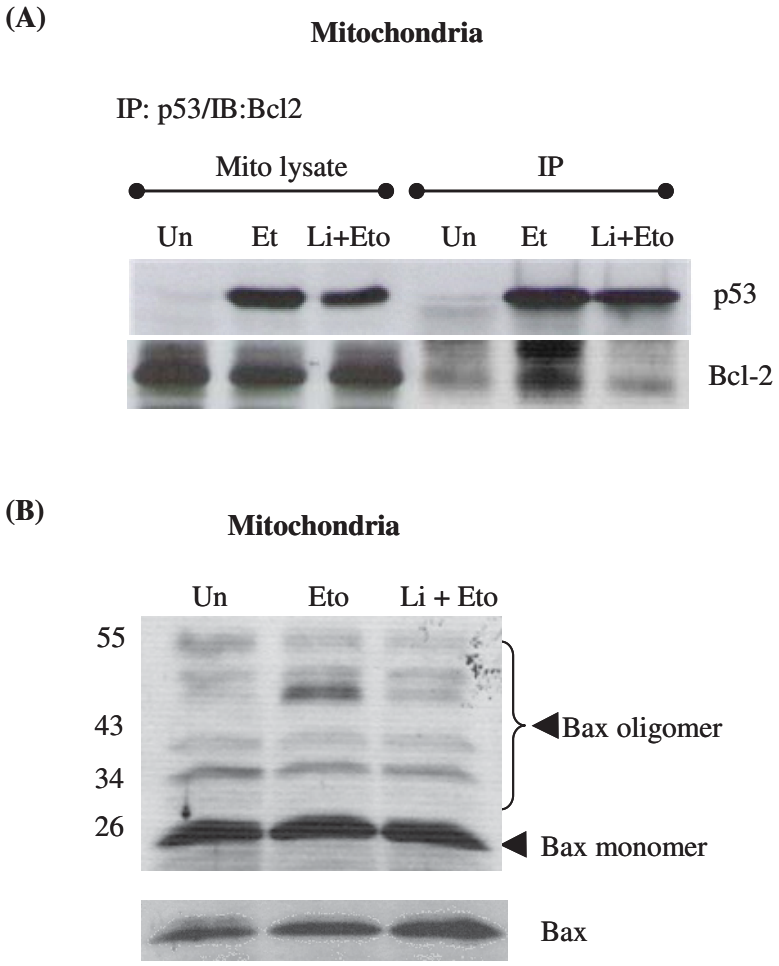

Figure 7. Inhibition of GSK3 reduced the interaction of p53 with Bcl2 and Bax oligomerization associated with mitochondria

SH-SY5Y cells were treated with 10 μM etoposide for 5 hr with or without a 30 min preincubation with the GSK3 inhibitor, lithium (20 mM). (A) Mitochondrial p53 was immunoprecipitated. The interaction of p53 and Bcl2 was detected by co-immunoprecipitation. (B) Mitochondria were lysed with 2% CHAPS lysis buffer followed by chemical cross-linking with EGS. Cross-linked proteins were resolved in 4-20% SDS-PAGE, and immunoblotted for Bax.

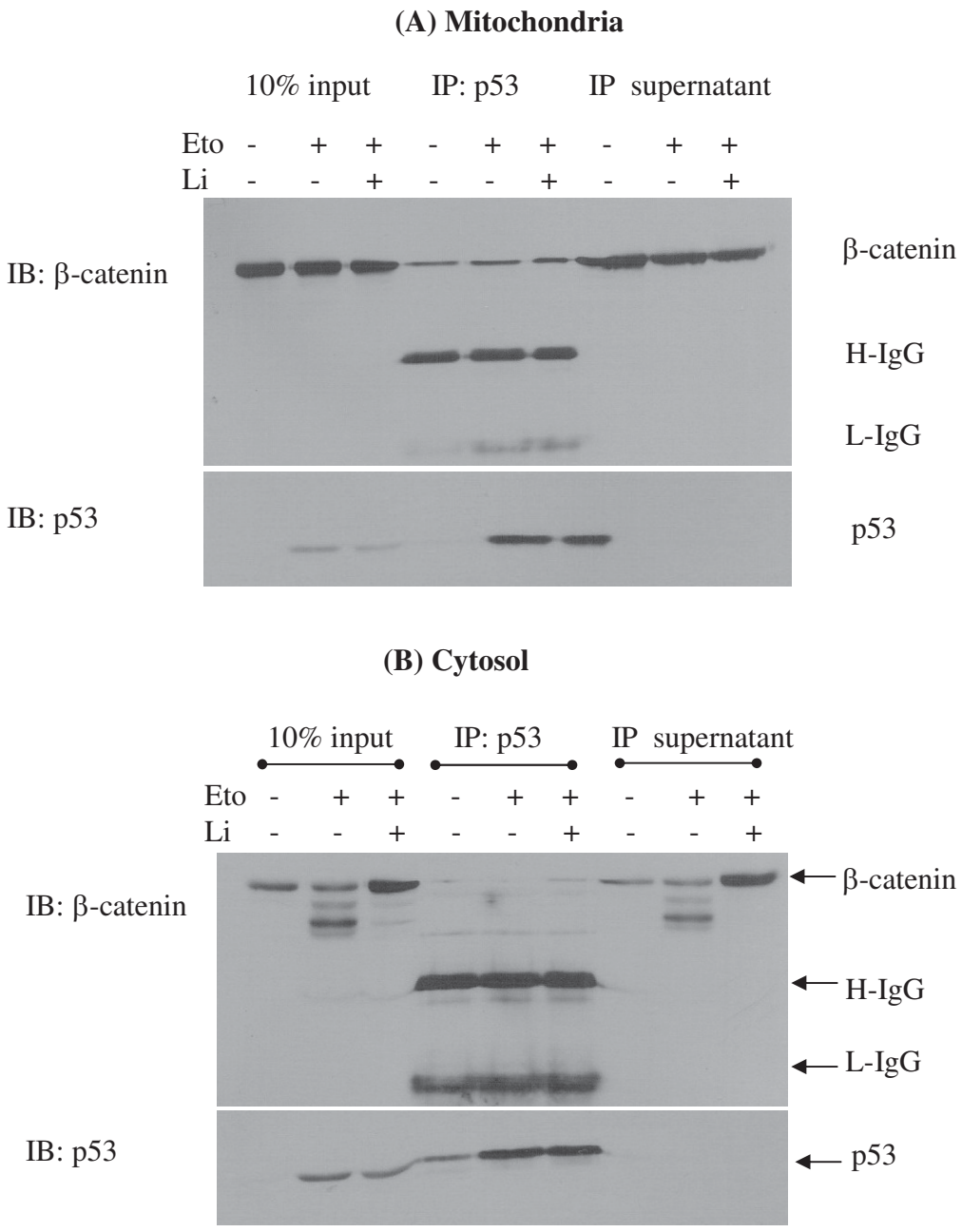

Figure 8. p53 co-immunoprecipitated with β-catenin.

SH-SY5y cells were treated with 10 μM etoposide with or without of lithium pretreatment. Mitochondrial and cytosolic p53 were immunoprecipitated and β-catenin was detected by immunoblot analysis to examine β-catenin-p53 co immunoprecipitation.

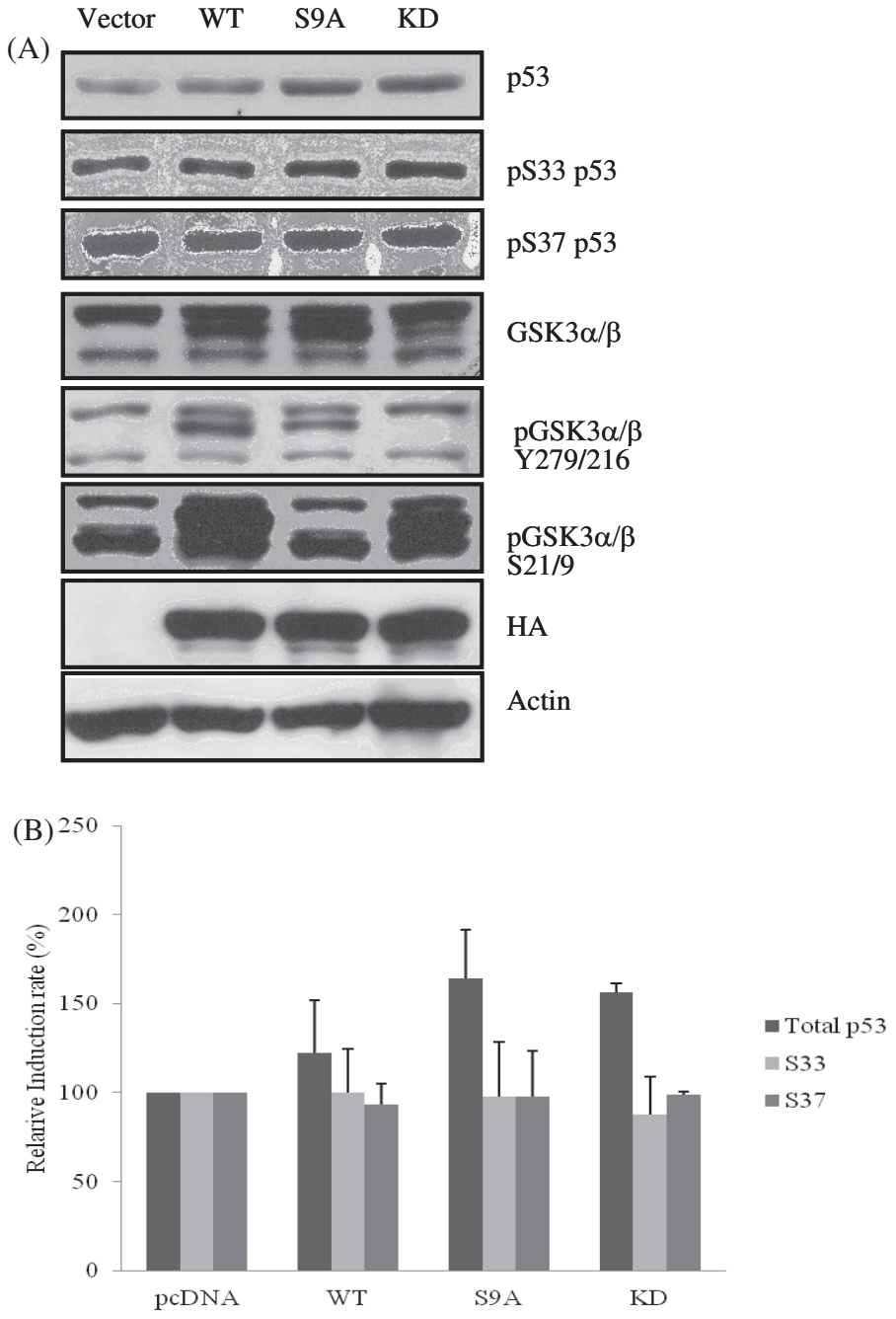

Figure 9. overexpression of GSK3β increases basal level of p53

Wild-type GSK3β (WT), constitute S9A mutant GSK3β (S9A), kinase dead GSK3β (KD) or vector control (pcDNA) was transfected in HEK293 cells. Levels of protein expression were determined by immunoblot analysis (A), and levels of p53, and phospho Serine-33 and 37 p53 were quantified by densitometric analysis (B).

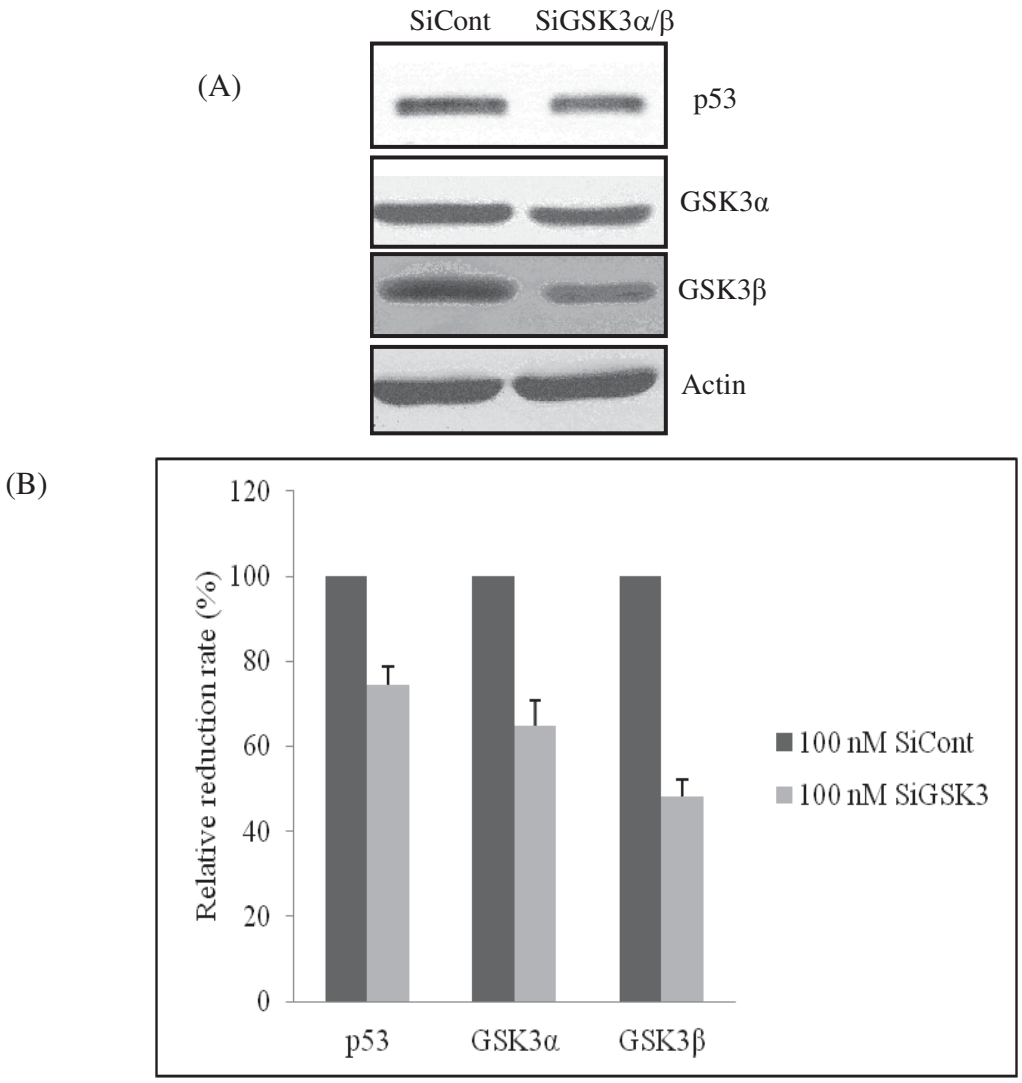

Figure 10. GSK3 contributes to maintaining p53 level in basal unstressed condition
HEK 293 cells were tranfected with siRNA GSK3α/β or SiRNA control. Levels of p53 GSK3α, and GSK3β were examined by western blotting (A), and quantitative analysis by densitrometry was presented as % control (B).

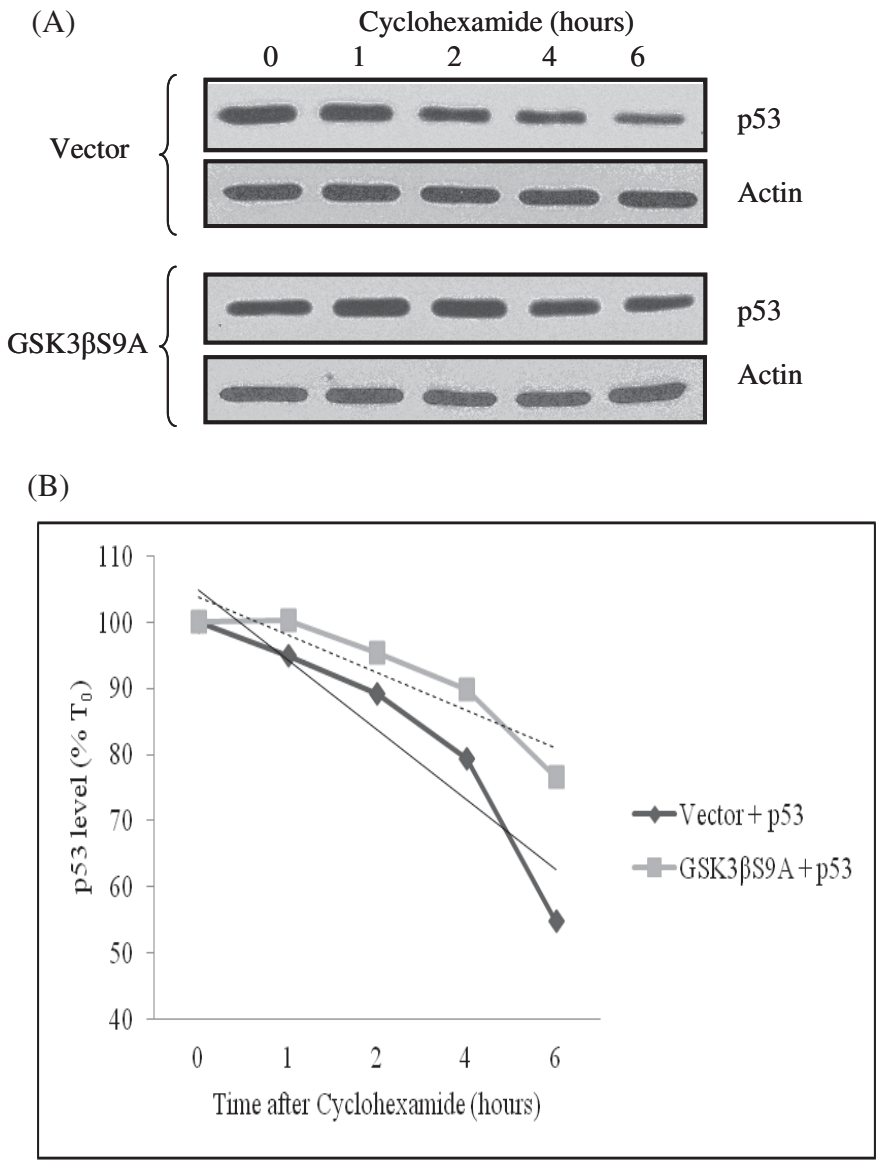

Figure 11. GSK3 overexpression increases p53 stability

p53 was co-transfected with GSK3βS9A or vector control in H1299 p53 null cells for 24 hours prior to addition of cyclohexamide (20 μM) to inhibit protein synthesis, and the incubation for indicated time. The loss of p53 expression over time was monitored by immunoblot analysis (A), and quantitative analysis by densitrometry was presented as % of cyclohexamide treatment at 0 hour (%T0) (B).

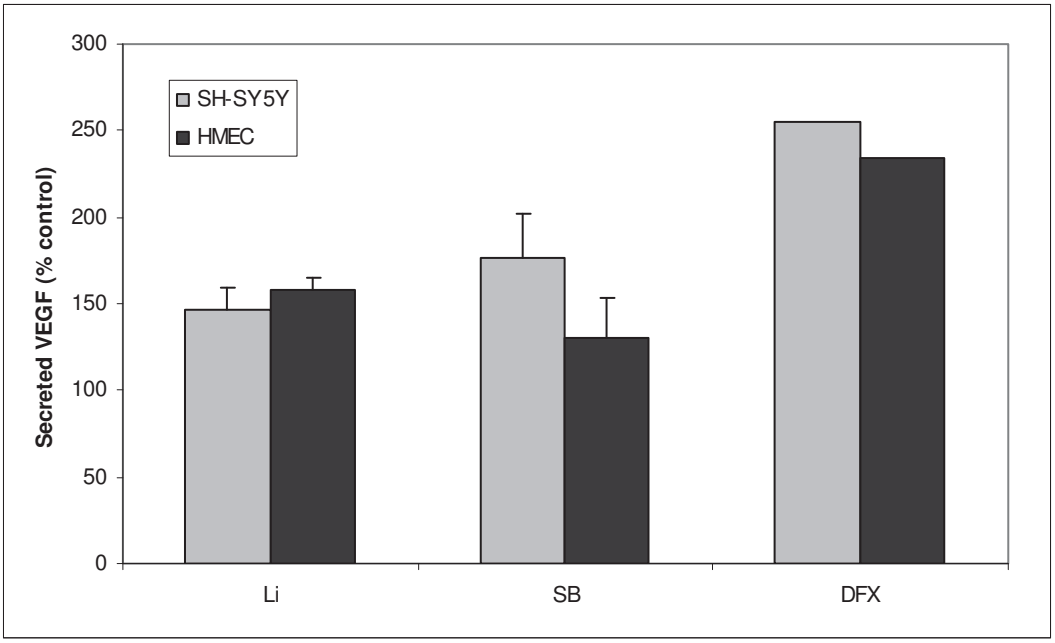

Figure 12. Inhibition of GSK3 caused increased in secreted VEGF in SH-SY5Y cells. SH-SY5Y cells or Human microendothelial cells (HMEC) were incubated in serum free medium with or without 20 mM lihitum (Li) or 20 μM SB216763 (SB) for 24 hours. Medium was collected for VEGF measurement by ELISA. 24 hours treatment with 100 μM desferoxamine, a hypoxia mimicking agent was used as a positive control. Data were presented as % of control.

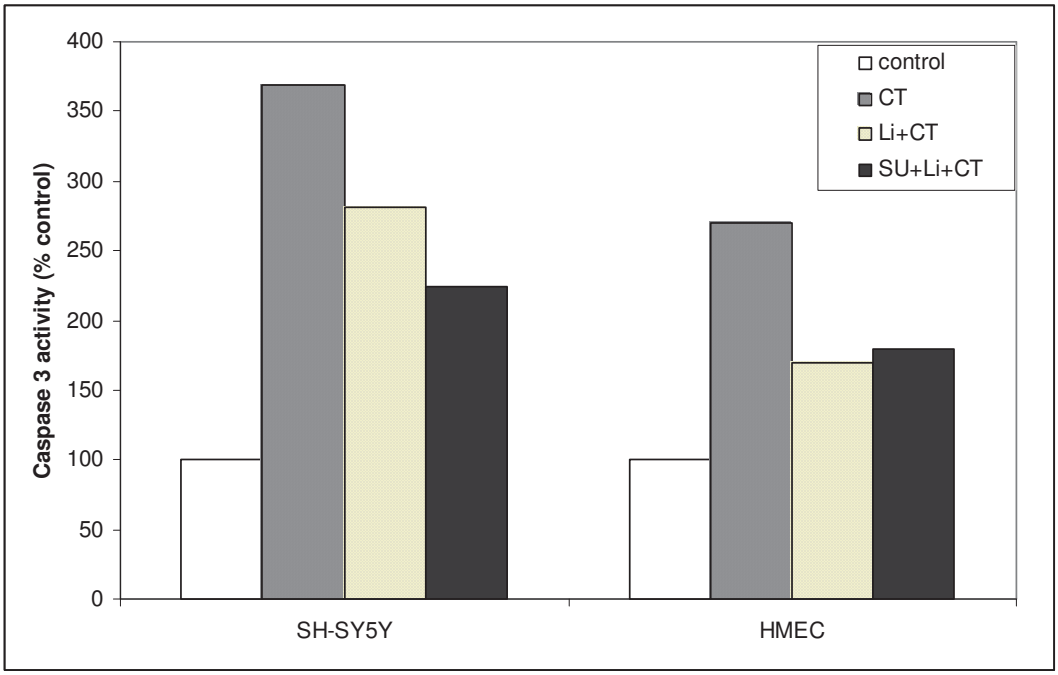

Figure 13. Inhibition of VEGF signaling could not reverse GSK3 inhibition protection against apoptosis.

SH-SY5Y and HMEC cells were treated with10 μM camptothecin, with or without pretreatment with lithium (20 mM) or lithium and SU4312 (12.5 μM). Apoptosis was determined by caspase 3 activation, and data were presented as % of control.

4. สรุปผลการวิจัย

Collectively, the present study reveals that 1) GSK3 promotes the mitochondrial translocation of wild-type but not mutated p53, where p53 interacts with Bcl2 to allow Bax oligomerization and the subsequent release of cytochrome C that leads to caspase activation in the mitochondrial pathway of intrinsic apoptotic signaling, 2) increase in GSK3 level augments p53 in basal condition through mechanism involves increase its stability, and GSK3 kinase activity was not required for this p53 induction effect 3) the antiapoptotic action of GSK3 inhibition may not involve its ability to suppress VEGF expression.

5. สรุปผลงานการดำเนินโครงการ

- การดำเนินการวิจัยบรรลุตามวัตถุประสงค์ข้อที่ 1 และ 2 ผลการทดลองสรุปได้ว่า GSK3 มีผลเสริม translocation ของ p53 ไปยัง mitochondria และ mitochondria pathway apoptosis อย่างไรก็ตามการทดลองหลายการทดลองที่ได้เสนอไว้ไม่สามารถดำเนินการได้โดยเฉพาะในส่วนของ molecular expression, organelle localization, และ colocalization ทั้งนี้เนื่องจากผู้วิจัยคาดหวังว่าจะใช้ immunofluorescent technigue และ inverted fluorescent microscope ในการตรวจสอบผลการทดลองดังกล่าว แต่เครื่องมือที่ผู้วิจัยมีอยู่ไม่มีประสิทธิภาพพอที่จะใช้ในการทดลองที่ผู้วิจัยคาดไว้ได้ ทำให้ผู้วิจัยไม่สามารถเก็บผลการทดลองที่จะนำไปสู่ข้อสรุปในเชิงลึกได้

-การวิจัยตามวัตถุประสงค์ข้อที่ 3 พบว่าผลการทดลองไม่เป็นได้ผลตามที่ผู้วิจัยคาดการไว้ในข้อเสนอโครงการที่ว่า การยับยั้ง VEGF signaling จะสามารถยับยั้งผลของ GSK3 inhibitor ในการต่อต้าน apoptosis ได้

6. ผลงานที่ได้จากงานวิจัย

-The findings from this research project provide further understanding in role of GSK3 in apoptosis that is GSK3 involves in mitochondrial pathway of apoptosis through regulation of p53 mitochondrial translocation. As apoptotic cell death is one of the key molecular mechanisms for chemotherapy, hence increase GSK3 activity may potentially enhance apoptotic action of chemotherapeutic agents against cancers barring wild-type p53 as we found that GSK3 only drives wild-type p53 translocation to the mitochondria.

-Poster presentation: The 3rd Biochemistry and Molecular Biology (BMB) Conference "From Basic to Translational Research for a Better Life", April 6-8, 2011, Chaingmai, Thailand

-Manuscript: Patchara Ngok-ngam, Piyajit Watcharasit, Apinya Thiantanawat, and Jutamaad Satayavivad. Pharmacologic modulation of GSK3 attenuated DNA damage-induced apoptosis via regulation of p53 mitochondrial translocation and Bax oligomerization in neuroblastoma SH-SY5Y cells. (In preparation) (Appendix 1)

ภาคผนวก 1

Manuscript

Pharmacologic modulation of GSK3 attenuated DNA damage-induced apoptosis via regulation of p53 mitochondrial translocation and Bax oligomerization in neuroblastoma SH-SY5Y cells

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Abstract
Glycogen synthase kinase-3 (GSK3) and p53 play crucial roles in the mitochondrial apoptotic pathway and are known to interact in the nucleus, but it is not known if GSK3 has a regulatory role in the mitochondrial translocation of p53 that participates in apoptotic signaling following DNA damage. Here we demonstrated that pharmacological inhibitors of GSK3, lithium and SB216763 attenuated p53 accumulation and caspase-3 activation induced by three mechanistically different DNA damaging agents, doxorubicin, etoposide, and camptothecin. Furthermore, each of these insults induced translocation of p53 to the mitochondria and mitochondrial pathway of apoptosis as evidence by the release of cytochrome c from mitochondria, and both of these apoptotic responses were attenuated by inhibition of GSK3 suggesting that GSK3 has promotional action on DNA damage-induced p53 mitochondrial translocation and mitochondrial apoptosis pathway. However, regulation of p53 mitochondrial translocation by GSK3 was only evident with wild-type, not mutated, p53. Additionally, inhibition of GSK3 reduced etoposide-induced association of p53 with Bcl2 and Bax oligomerization. Taken together, our findings reveal that GSK3 promotes the mitochondrial translocation of p53, where it interacts with Bcl2 to allow Bax oligomerization and the subsequent release of cytochrome C that leads to caspase activation in the mitochondrial pathway of intrinsic apoptotic signaling.

Keywords: GSK3, p53, mitochondria translocation, apoptosis, Bax oligomerization

Introduction

GSK3 is a multifunctional serine/threonine kinase that plays critical roles in the regulation of multiple signaling pathways, including apoptotic signaling (1). GSK3 has been shown to promote apoptotic cell death induced by several insults that induce apoptosis, such as DNA damage (2), endoplasmic reticulum stress (3-5) and mitochondrial toxins (6-7). Overexpression of GSK3β is sufficient to cause apoptosis in a manner dependent on p53, which is one of the key components in apoptosis signaling particularly following DNA damage (8). Additionally, a direct interaction between GSK3 and p53 has been demonstrated in the nucleus, and this interaction increased GSK3 activity in the nucleus and promoted the apoptotic action of p53 (2, 9). Moreover, GSK3 phosphorylates p53 at serines 33 (10), 315 and 376 (5), which are sites that, after phosphorylation, increase the apoptotic action of p53. It is interesting to note that serine-376 of p53 is within the region that has been identified to be necessary for the interaction of p53 with GSK3 (9). Taken together, these findings suggest that the interaction of GSK3 with p53 may be important for controlling p53-mediated apoptosis.

It is well recognized that the tumor suppressor protein, p53 which its mutations are found in approximately 50% of human cancers causes apoptosis. In part, p53 mediates apoptosis through transcriptionally promoting expression of proapoptotic proteins such as Bax and PUMA (11). Additionally, several lines of evidence demonstrated that p53 is capable of inducing apoptosis independently of its transcriptional activity via its translocation to the mitochondria (12-16). Interestingly, GSK3 has been shown to promote the intrinsic pathway of apoptotic signaling in which mitochondria have a critical role (1). The present study was designed to further investigate the role of GSK3 modulation on the translocation of p53 to the mitochondria and p53 transcriptional-independent mitochondrial apoptotic action. The results show that GSK3 controls p53 mitochondrial translocation, as well as mitochondria-mediated apoptosis, in part by promoting p53 mitochondrial translocation and its interaction with Bcl2 that allows Bax oligomerization and subsequently cytochrome C release and apoptosis.

Materials and methods

Cell culture: SH-SY5Y human neuroblastoma cells (ATCC) were maintained in MEM:Ham's F12 (1:1) media (Gibco) supplemented with 10% FBS (JR Scientific Inc.), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco), and cultured in 5% CO_2 at 37 ℃. T47D cells (ATCC) were cultured in RPMI 1690 media supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 20 mM D-glucose, 1 mM sodium pyruvate (Gibco) and 13.9 mM insulin (Sigma-Aldrich).

Subcellular fractionation*:* Subcellular fractionation was performed as we described previously (17) . Briefly, cells were incubated with extraction buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.02% digitonin, 0.1 mM PMSF, 1mM Na_3VO_4, 20 mM NaF and protein inhibitor cocktail) for 10 min on ice. The cell extracts were then spun at 700×g for 5 min at 4℃ resulting in pellet containing nucleus and supernatant containing cytsol and mitochondria. The pellet of the nuclear fraction was then washed with wash buffer twice (10 mM HEPES pH 7.4, 10 mM NaCl, 250 mM sucrose, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1 mM Na_3VO_4, 20 mM NaF and protease inhibitor cocktail) and lysed with nuclear lysis buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 300 mM NaCl, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 50 mM NaF, and protease inhibitor cocktail) for 30 min at 4℃. The nuclear extracts were clarified by centrifugation at 16000×g, 15 min, 4℃. The supernatant was kept as the nuclear fraction. The supernatant containing cytosol and mitochondria was centrifuged at 16000×g for 30 min at 4℃ to collect the pellet enriched mitochondrial and the supernatant cytosolic

fractions. The mitochondria were washed twice with wash buffer, and lysed by incubation with lysis buffer for 30 minutes on ice. The mitochondrial lysates were clarified by centrifugation at 16000×g for 15 minutes at 4℃. The cytosol was subjected to ultracentrifugation under 110000×g, 30 min at 4℃ to collect the supernatant cytosolic fraction.

Immunoblot analysis: Cells were lysed with lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 % Nonidet-P40, 0.1 mM PMSF, 1 mM Na_3VO_4, 20 mM NaF and proteinase cocktail inhibitor (Calbiochem)). Cell lysates were sonicated and then centrifuged at 20,000×g for 15 min at 4℃. Protein concentration was determined by using the Bradford reagent (Bio-Rad). Immunoblotting was performed as described previously (17). The antibodies to GSK3α/β, phospho-tyrosine279/216-GSK3α/β, p53, Bcl2 and Bax were purchased from Millipore, PARP, p21 and β-tubulin were from BD Pharmingen Bioscience, cytochrome C, ATP synthase F1 subunit and histone H1 were from Santa Cruz Biotechnology, phospho-serine21/9-GSK3α/β was from Cell Signaling Technology, and β-actin was from Sigma-Aldrich. For immunoprecipitation, 60 μg of mitochondrial protein was incubated with 1 μg of p53 antibody for 2 hr at 4℃ with gentle agitation followed by incubation with protein G-sepharose beads (GE Healthcare) for 2 hr at 4℃. The immune complexes were washed, mixed with Laemmli sample buffer (2% SDS) and boiled for 10 min. The eluted protein complexes were subjected to immunoblot analysis using anti-p53 and Bcl2 antibodies. All experiments were repeated three or more times.

Chemical cross-linking: Mitochondrial protein chemical cross-linking was performed as described in (17) using a bifunctional cross-linker, ethylene glycol bis(sulfosuccinimidyl succinate) (EGS, 1mM) (Pierce). The cross-linked samples were resolved in 4-20% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membrane was probed with anti-Bax antibody. At least three independent experiments were completed for each treatment.

Results

GSK3 inhibition attenuates DNA damage-induced increased p53 levels and caspase activation

To study p53-mediated apoptosis signaling, we used three mechanistically different DNA damaging agents that induce p53 expression: doxorubicin, a DNA intercalating agent, etoposide, a topoisomerase II inhibitor, and camptothecin, a topoisomerase I inhibitor. Treatment of human neuroblastoma SH-SY5Y cells, which express wild-type p53, with doxorubicin, etoposide, or camptothecin caused large increases in level of p53 (Fig.1). Activation of apoptosis was evident by increases in the cleavage of PARP, which is a substrate of caspase 3, a key enzyme activated during apoptosis (Fig. 1). Levels of the anti-apoptotic protein, Bcl2, and the pro-apoptotic protein, Bax, were not affected by doxorubicin, etoposide or camptothecin treatments (Fig.1), suggesting that apoptosis induced by the three inducers of DNA damage was not due to imbalances of these proteins.

To test whether GSK3 is involved in the regulation of p53 induction and apoptosis, two mechanistically different selective inhibitors of GSK3, lithium and SB216763, were used. GSK3 activity is mainly regulated by phosphorylation, with activity inhibited by N-terminal serine-phosphorylation (serine-21 for GSK3α and serine-9 for GSK3β), and activity increased by tyrosine phosphorylation (tyrosine-279 for GSK3α and tyrosine-216 for GSK3β) (1). GSK3 inhibition by lithium caused increases in the serine phosphorylation of both GSK3 isoforms, as reported previously (18-19). SB216763 directly inhibits GSK3 by competition at the ATP binding site (20), which resulted in decreases of both serine and tyrosine-phosphorylation of GSK3α/β (Fig. 1). Pretreatment with lithium or SB216763 reduced the induction p53 and cleavage of PARP induced by treatment with doxorubicin, etoposide and camptothecin (Fig. 1), indicating that GSK3 contributes to the induced expression of p53 and

caspase activation caused by each of these DNA damaging agents. However, lithium and SB216763 did not alter the levels of Bcl2 or Bax (Fig. 1), indicating that the apoptotic protection by GSK3 inhibition is unlikely to be through modulation of the levels of Bcl2 or Bax.

Inhibition of GSK3 reduces p53 translocation to mitochondria and apoptosis

Since translocation of p53 to the mitochondria is capable of inducing apoptosis (21), we investigated the contribution of GSK3 to p53 mitochondrial translocation and its activation of mitochondria-mediated apoptosis. SH-SY5Y cells were treated with doxorubicin (2 μM), etoposide (10 μM) or camptothecin (5 μM) for 5 hr, and mitochondrial, cytosolic, and nuclear fractions were prepared. The purity of the mitochondrial, cytosolic and nuclear preparation was verified by immunoblotting for a marker of each fraction, ATP synthase F1 subunit, β-tubulin and histone H1, respectively (Fig. 2A). Treatment with doxorubicin, etoposide, or camptothecin caused significant increases in the levels of p53 in the mitochondria (Fig. 2B), and cytochrome C release into the cytosol, although cytochrome c release after camptothecin treatment was mild at this time point (Fig. 3), which indicates activation of the mitochondrial apoptosis pathway. Inhibition of GSK3 by lithium or SB216763 prominently attenuated p53 translocation to the mitochondria following doxorubicin, etoposide and camptothecin treatment (Fig. 2B). Quantitative analysis revealed that inhibition of GSK3 with lithium or SB216763 significantly reduced DNA damage-induced p53 translocation to mitochondria (Fig. 2C). Treatment with the GSK3 inhibitors did not alter the levels of p53 in the cytosol and nucleus (Fig. 2B). These results indicate that active GSK3 contributes to p53 mitochondrial translocation after DNA damage. Additionally, GSK3 inhibition reduced cytochrome C release (Fig. 3). These results indicate that GSK3 promotes both p53 mitochondrial translocation and the mitochondrial apoptosis pathway after DNA damage.

GSK3 regulates mitochondrial translocation of wild-type but not mutant p53

It has been reported that some transcriptionally incompetent mutants of p53 are capable of inducing apoptosis (22-23) and are present in the mitochondria after induction of apoptosis (14, 24). Therefore, we investigated whether GSK3 controls mitochondrial translocation of mutant p53. To do this, breast cancer T47D cells expressing L194F mutant p53 (25) were used. Unlike wild-type p53, which is expressed at very low levels in unstressed cells such as SH-SY5Y cells, mutant p53 is expressed at high levels in untreated T47D cells (Fig. 4A). Additionally, we found higher levels of mutant p53 than wild-type p53 are in the mitochondria of untreated cells (Fig. 4A). Inhibition of GSK3 with lithium did not affect the basal levels of mutant p53 in the mitochondria in T47D cells (Fig. 4B). Moreover, treatment with etoposide did not significantly affect the mitochondrial levels of mutant p53, and GSK3 inhibition did not alter mitochondrial mutant p53 levels following treatment with etoposide (Fig. 4C). These results indicate that GSK3 only regulates the mitochondrial translocation of wild-type p53, but not mutant p53, or that GSK3 only regulates the increase in mitochondrial p53 that follows activation of apoptotic signaling, which was absent in T47D cells.

Inhibition of GSK3 reduces the interaction of p53 with Bcl2 and Bax oligomerization

It has been shown that p53 directly induces mitochondrial apoptosis through its interaction with Bcl2, a key protein regulating the mitochondrial outer membrane permeability (26). Thus, we examined the p53-Bcl2 interaction in mitochondria by co-immunoprecipitation. The level of p53 in mitochondria was increased, whereas level of Bcl2 was unaltered, by etoposide treatment (Fig. 5A). Co-immunoprecipitation of p53 and Bcl2 was observed in etoposide-treated cells, and this p53-Bcl2 interaction was drastically reduced by treatment with the GSK3 inhibitor lithium (Fig. 5A). We further investigated whether GSK3 inhibition affected Bax oligomerization, which can lead to

mitochondrial outer membrane permeabilization and subsequently cytochrome C release. To detect Bax oligomerization, after treatments mitochondrial proteins were cross-linked by treatment with EGS, followed by western blot analysis. The results show that etoposide treatment increased the immunoreactivity of a slow migrating band above the 21 kD Bax monomer band (Fig. 5B) indicating increased Bax oligomerization associated with the mitochondria following etoposide treatment. Inhibition of GSK3 with lithium treatment attenuated etoposide-induced Bax oligomerization in the mitochondria. The level of Bax in the mitochondria was not altered by either etoposide or lithium, indicating that the reduction of Bax oligomerization by GSK3 inhibition was not due to a decrease of Bax in the mitochondria.

Taken together, these results indicate that GSK3 regulates mitochondrial apoptotic signaling in part by facilitating p53 translocation to the mitochondria where it binds and neutralizes the anti-apoptotic protein Bcl2, allowing Bax oligomerization that increases mitochondria permeabilization and cytochrome C release.

Discussion

Considering the recently identified direct action of p53 in mitochondria in inducing apoptosis (26-27), and the direct, activating interaction between p53 and GSK3 in the mitochondrial pathway of apoptosis (2, 9), we tested if GSK3 plays a role in regulating mitochondrial p53. The findings in this investigation reveal that after DNA damage GSK3 promotes p53 translocation to mitochondria, Bax oligomerization associated with the mitochondria, and mitochondria-mediated apoptotic signaling. The results indicate that GSK3 facilitates the mitochondrial pathway of apoptosis in part through promoting p53 translocation to the mitochondria where it binds and sequesters the anti-apoptotic protein, Bcl2, which allows Bax oligomerization that leads to release of cytochrome C from the mitochondria to promote caspase activation.

The apoptotic activity of p53 has been demonstrated to include both transcriptional-dependent and -independent actions. The transcriptional-dependent apoptotic action of p53 is well-characterized to be mediated by transactivation of pro-apoptotic genes including Bax and PUMA (11), and transrepression of antiapoptotic genes, such as Bcl2 and survivin (11). The transcriptional-independent apoptotic action of p53 was identified by overexpression of transcriptionally deficient p53 mutants that were capable of triggering apoptosis (23). Recently, it has been shown that expression of mitochondria-targeted p53 caused apoptosis in p53-null cells (13-14, 21), indicating that transcription-independent p53-mediated apoptosis occurred via its action in mitochondria. Recently, we reported that inhibition of GSK3 reduced p53 translocation to the mitochondria and mitochondrial apoptotic cell death following arsenite treatment, suggesting a regulatory role for GSK3 in the translocation of p53 to the mitochondria and the mitochondrial apoptotic pathway (17). The present study extended these finding by demonstrating that inhibition of GSK3 by two structurally unrelated selective GSK3 inhibitors, lithium and SB216763, attenuated p53 translocation to the mitochondria induced by three different DNA damaging agents that induce apoptosis by activating p53, namely doxorubicin, etoposide and camptothecin, in SH-SY5Y cells. Since mutant p53 was reported to reside in the mitochondria, we also examined the regulation by GSK3 of mitochondrial translocation of mutant p53. We observed that in T47D cells expressing mutant p53 (p53L194F), basal mitochondrial p53 levels were relatively high, which is consistent with previous reports (14, 24). Unlike wild-type p53, inhibition of GSK3 did not affect the mitochondrial level of mutant p53, with or without an apoptotic stimulus, suggesting that GSK3 only regulates the translocation of wild-type p53 to the mitochondria. Although recent studies have demonstrated the importance of mitochondrial p53 in the mitochondrial pathway of apoptosis, little is known about the mechanisms that regulate its translocation. These findings add GSK3 to known regulators of p53 mitochondrial

translocation, which also includes the previously identified mono-ubiquitination (28) and Tid1 (mtHSP40) (24). The findings in the present study also indicate that GSK3 only regulates the mitochondrial translocation of wild-type p53, but not mutant p53. Regarding the mechanism by which GSK3 regulates p53 translocation to the mitochondria, we speculate that it may be independent of GSK3-mediated p53 phosphorylation as p53 mitochondrial translocation has been demonstrated to be independent of its phosphorylation state (29). Mechanistic insights GSK3 regulation of p53 mitochondrial translocation will require furhter analysis. The apoptosis promoting action of GSK3 has been demonstrated previously, showing that GSK3 promotes the intrinsic mitochondrial pathway of apoptosis but not the extrinsic pathway of apoptosis (1). The finding that GSK3 promotes p53 translocation to the mitochondria, which has been shown to induce mitochondrial apoptosis, led us to examine the contribution of GSK3 to the actions of p53 in mitochondria. In the mitochondria, p53 was reported to form complexes with Bcl2 and Bcl-xl, antiapoptotic members of the Bcl2 family that play critical roles in controlling the mitochondrial outer membrane permeabilization (MOMP). Consequently, this interaction of p53 with Bcl2/Bcl-xl induced MOMP, leading to cytochrome C release and subsequently apoptosis (14). Our study also found that p53 interacts with Bcl2 following etoposide treatment, and inhibition of GSK3 reduced this interaction. However, we were unable to detect the interaction of p53 with Bcl-xL in these cells. Additionally, we found that inhibition of GSK3 reduced etoposide-induced Bax oligomerization, which leads to MOMP. Bax oligomerization results from disruption of Bcl2-Bax complexes that inhibit MOMP following the sequestration of Bcl2 by p53. p53 has been shown to form a pore complex with Bax, suggesting the potential for a direct interaction with Bax in triggering MOMP (27). However, we did not detect p53 complexed with oligomerized Bax in our experiment paradigm, which is consistence with a previous report (14). Interestingly, although Bax phosphorylation by GSK3 has been shown to increase its translocation to mitochondria (30), GSK3-meidiated Bax phosphorylation is unlikely to be critical for Bax mitochondrial translocation in our experimental conditions as the level of Bax in mitochondria was not affected by GSK3 inhibition (Fig 5B).

It is clearly evident that mitochondrial p53 can induce apoptosis independent of its transcriptional activity, and induction of apoptosis is one of the major mechanistic targets for chemotherapeutic agents especially those cause DNA damage. Extended from our finding that GSK3 promotes DNA damage-induced p53 mitochondrial translocation and mitochondrial pathway of apoptosis, enhanced GSK3 activity may be beneficial for chemotherapy of cancer harboring wild-type p53. Moreover, mitochondrial p53 was demonstrated experimentally to have tumor suppressor activity in vivo as expression of mitochondrial targeted p53 suppressed tumor growth in p53 null lymphoma xenograft mice (31). Hence, augmentation of p53 level in the mitochondria by GSK3 activation may improve p53 tumor suppressor action. It is interesting to note that generally, under normal physiological condition GSK3 activity is suppressed in response a number of cellular signaling including growth factor mediated Akt activation and insulin signaling. Indeed, GSK3 activity possibly increases by inactivation of Akt by growth factor withdrawal and inhibition of insulin signaling, andrapamycin treatment (32). However, it remains to be investigated whether increase in GSK3 activity will be sufficient to enhance p53 mitochondrial translocation in unstressed condition.

In summary, this study found that inhibition of GSK3 reduced wild-type, but not mutant, p53 mitochondrial translocation, the mitochondrial interaction of p53 with Bcl2, and Bax oligomerization concomitant with a reduction of cytochrome C release. These results indicate that GSK3 promotes mitochondrial apoptosis partly through facilitating p53 translocation to mitochondria where it binds and neutralizes Bcl2, thus allowing

Bax oligomerization and the subsequent release of cytochrome C release in the mitochondrial apoptotic signaling pathway.

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Conflict of Interests

The authors declare no conflict of interest.

References

1. Beurel E and Jope RS: The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. Prog Neurobiol 79: 173-189, 2006.

2. Watcharasit P, Bijur GN, Zmijewski JW, Song L, Zmijewska A, Chen X, Johnson GV and Jope RS: Direct, activating interaction between glycogen synthase kinase-3beta and p53 after DNA damage. Proc Natl Acad Sci U S A 99: 7951-79555, 2002.

3. Kim AJ, Shi Y, Austin RC and Werstuck GH: Valproate protects cells from ER stress-induced lipid accumulation and apoptosis by inhibiting glycogen synthase kinase-3. J Cell Sci 118: 89-99, 2005.

4. Song L, De Sarno P and Jope RS: Central role of glycogen synthase kinase-3beta in endoplasmic reticulum stress-induced caspase-3 activation. J Biol Chem 277: 44701-44708, 2002.

5. Qu L, Huang S, Baltzis D, Rivas-Estilla AM, Pluquet O, Hatzoglou M, Koumenis C, Taya Y, Yoshimura A and Koromilas AE: Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta. Genes Dev 18: 261-277, 2004.

6. King TD, Bijur GN and Jope RS: Caspase-3 activation induced by inhibition of mitochondrial complex I is facilitated by glycogen synthase kinase-3beta and attenuated by lithium. Brain Res 919: 106-114, 2001.

7. King TD and Jope RS: Inhibition of glycogen synthase kinase-3 protects cells from intrinsic but not extrinsic oxidative stress. Neuroreport 16: 597-601, 2005.

8. Pap M and Cooper GM: Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem 273: 19929-19932, 1998.

9. Watcharasit P, Bijur GN, Song L, Zhu J, Chen X and Jope RS: Glycogen synthase kinase-3beta (GSK3beta) binds to and promotes the actions of p53. J Biol Chem 278: 48872-9, 2003.

10. Turenne GA and Price BD: Glycogen synthase kinase3 beta phosphorylates serine 33 of p53 and activates p53's transcriptional activity. BMC Cell Biol 2: 12, 2001.

11. Harms K, Nozell S and Chen X: The common and distinct target genes of the p53 family transcription factors. Cell Mol Life Sci 61: 822-842, 2004.

12. Dumont P, Leu JI, Della Pietra AC, 3rd, George DL and Murphy M: The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet 33: 357-365, 2003.

13. Marchenko ND, Zaika A and Moll UM: Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J Biol Chem 275: 16202-16212, 2000.

14. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P and Moll UM: p53 has a direct apoptogenic role at the mitochondria. Mol Cell 11: 577-590, 2003.

15. Moll UM, Wolff S, Speidel D and Deppert W: Transcription-independent pro-apoptotic functions of p53. Curr Opin Cell Biol 17: 631-636, 2005.

16. Murphy ME, Leu JI and George DL: p53 moves to mitochondria: a turn on the path to apoptosis. Cell Cycle 3: 836-839, 2004.
17. Watcharasit P, Thiantanawat A and Satayavivad J: GSK3 promotes arsenite-induced apoptosis via facilitation of mitochondria disruption. J Appl Toxicol 28: 466-474, 2008.
18. Chalecka-Franaszek E and Chuang DM: Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. Proc Natl Acad Sci U S A 96: 8745-8750, 1999.
19. De Sarno P, Li X and Jope RS: Regulation of Akt and glycogen synthase kinase-3 beta phosphorylation by sodium valproate and lithium. Neuropharmacology 43: 1158-1164, 2002.
20. Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Brown MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD and Holder JC: Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chem Biol 7: 793-803, 2000.
21. Erster S, Mihara M, Kim RH, Petrenko O and Moll UM: In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. Mol Cell Biol 24: 6728-6741, 2004.
22. Chen X, Ko LJ, Jayaraman L and Prives C: p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev 10: 2438-2451, 1996.
23. Haupt Y, Rowan S, Shaulian E, Vousden KH and Oren M: Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev 9: 2170-2183, 1995.
24. Ahn BY, Trinh DL, Zajchowski LD, Lee B, Elwi AN and Kim SW: Tid1 is a new regulator of p53 mitochondrial translocation and apoptosis in cancer. Oncogene 29: 1155-1166, 2010.
25. O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Weinstein JN, Friend S, Fornace AJ, Jr. and Kohn KW: Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res 57: 4285-4300, 1997.
26. Vaseva AV and Moll UM: The mitochondrial p53 pathway. Biochim Biophys Acta 1787: 414-420, 2009.
27. Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M and Green DR: Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303: 1010-1014, 2004.
28. Marchenko ND, Wolff S, Erster S, Becker K and Moll UM: Monoubiquitylation promotes mitochondrial p53 translocation. Embo J 26: 923-934, 2007.
29. Nemajerova A, Erster S, & Moll UM. The post-translational phosphorylation and acetylation modification profile is not the determining factor in targeting endogenous stress-induced p53 to mitochondria. Cell Death Differ 12(2):197-200, 2005.
30. Linseman DA, et al. Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. J Neurosci 24(44):9993-10002, 2004.
31. Talos F, Petrenko O, Mena P, & Moll UM Mitochondrially targeted p53 has tumor suppressor activities in vivo. Cancer Res 65(21):9971-9981, 2005.
32. Dong J, et al. Role of glycogen synthase kinase 3beta in rapamycin-mediated cell cycle regulation and chemosensitivity. Cancer Res 65(5):1961-1972, 2005.

Figure legends

Figure 1. GSK3 inhibition attenuates DNA damage-induced increased p53 levels and caspase activation SH-SY5Y cells were treated with doxorubicin (Dox, 2 μM), etoposide (10 μM, Eto), or camptothecin (CT, 5 μM) for 5 hr with or without a 30 min pretreatment with GSK3 inhibitors, lithium (Li, 20 mM) or SB216763 (SB, 20 μM). (A) Levels of p53, PARP, pS21/9-GSK3α/β, pY279/216-GSK3α/β, GSK3α/β, Bcl2, and Bax were analyzed by immunoblot analysis and β-actin was used as a loading control. Caspase activation was determined by measuring PARP proteolysis.

Figure 2. Inhibition of GSK3 reduced p53 mitochondrial translocation
The mitochondrial, nuclear, and cytosolic fractions were prepared from cells treated with doxorubicin, etoposide, or camptothecin for 5 hr with or without a 30 min pretreatment with GSK3 inhibitors, lithium or SB216763. (A) Mitochondria (Mito), cytosolic (Cyto), and nuclear (Nuc) fractions were immunoblotted with a protein marker for each fraction, the F1 subunit ATP synthase for mitochondria, β-tubulin for the cytosol, and histone H1 for the nucleus to verify the purity of each fraction. (B) The levels of mitochondrial, cytosolic and nuclear p53 were detected by immunoblotting. (C) The quantitative data of mitochondrial p53 were expressed as % individual chemical treatment without GSK3 inhibition (mean ± SD; n=3), * $p < 0.05$.

Figure 3. Inhibition of GSK3 reduced cytochrome c release from the mitochondria
SH-SY5Y cells were treated with doxorubicin (Dox, 2 μM), etoposide (10 μM, Eto), or camptothecin (CT, 5 μM) for 5 hr with or without a 30 min pretreatment with GSK3 inhibitors, lithium (Li, 20 mM) or SB216763 (SB, 20 μM), and cytochrome C released into the cytosol was measured by immunoblotting.

Figure 4. GSK3 regulates the mitochondrial translocation of wild-type but not mutant p53
(A) Levels of mitochondrial p53 in SH-SY5Y (wild-type p53) and T47D (L194F p53) cells. (B) T47D cells were treated with 20 mM lithium for 1, 3 or 5 hr and the levels of mitochondrial p53 were measured by immunoblot analysis. (C) T47D cells were treated with etoposide (10 μM) with or without 20 mM lithium for 24 hr and the levels of mitochondrial p53 were analyzed by immunoblotting.

Figure 5**.** Inhibition of GSK3 reduced the interaction of p53 with Bcl2 and Bax oligomerization associated with mitochondria
SH-SY5Y cells were treated with 10 μM etoposide for 5 hr with or without a 30 min preincubation with the GSK3 inhibitor, lithium (20 mM). (A) Mitochondrial p53 was immunoprecipitated. The interaction of p53 and Bcl2 was detected by co-immunoprecipitation. (B) Mitochondria were lysed with 2% CHAPS lysis buffer followed by chemical cross-linking with EGS. Cross-linked proteins were resolved in 4-20% SDS-PAGE, and immunoblotted for Bax.

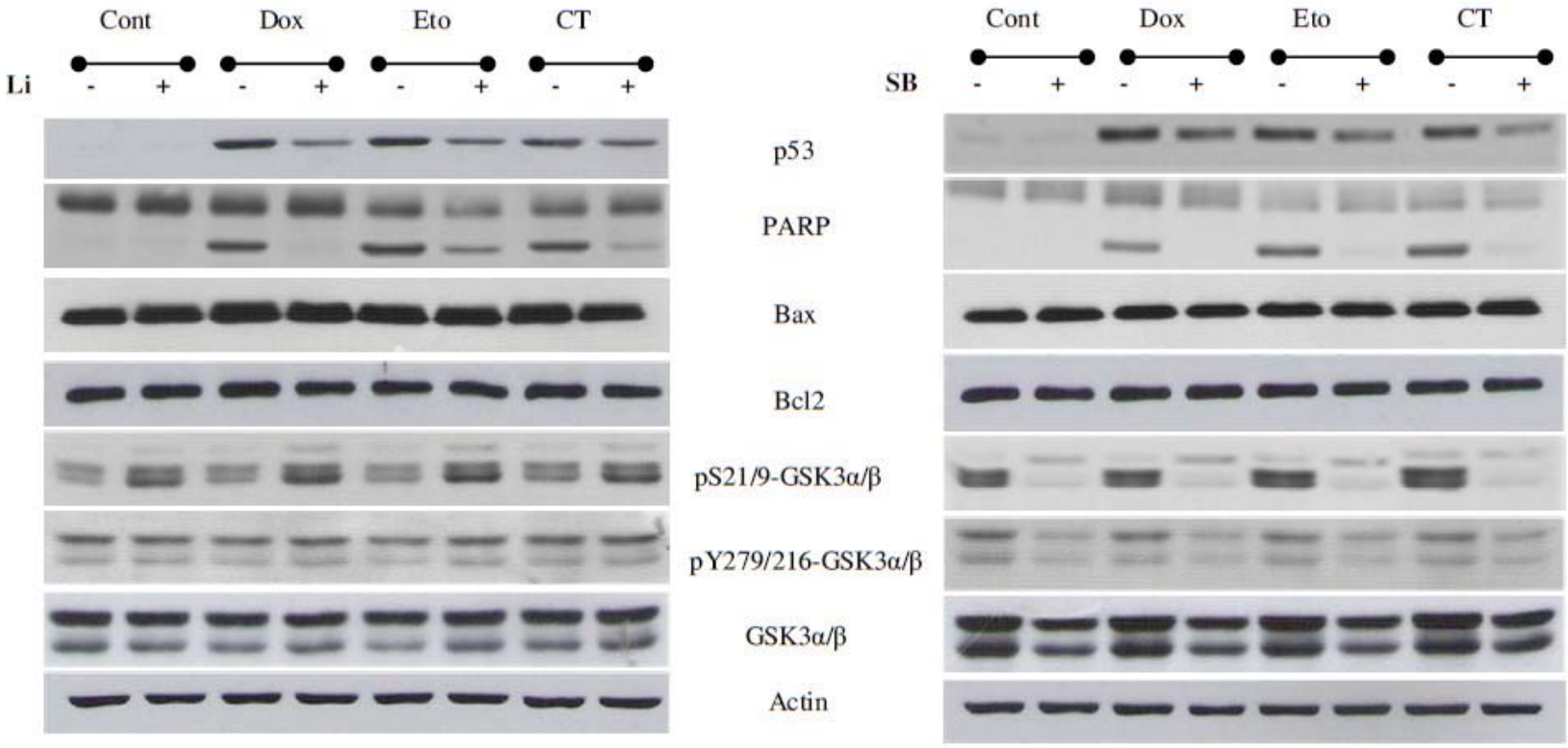
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Figure 1

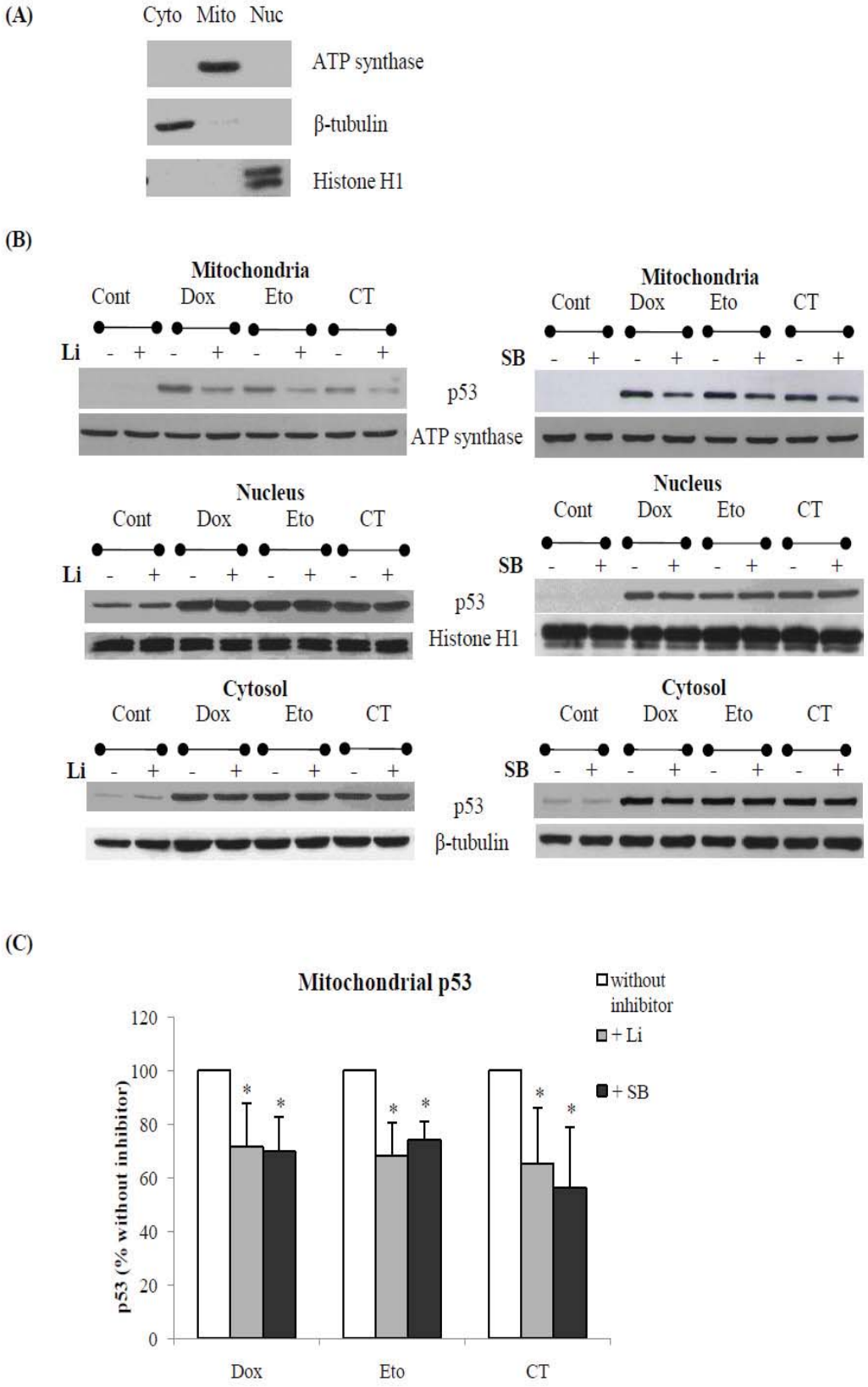
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Figure 2

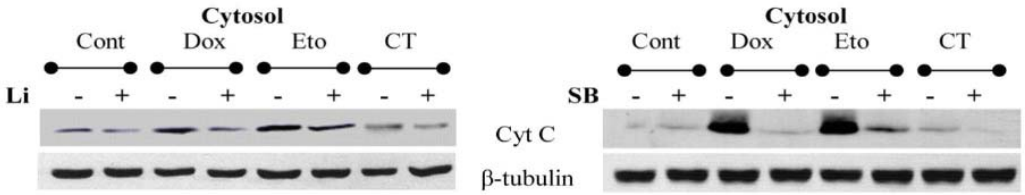
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Figure 3

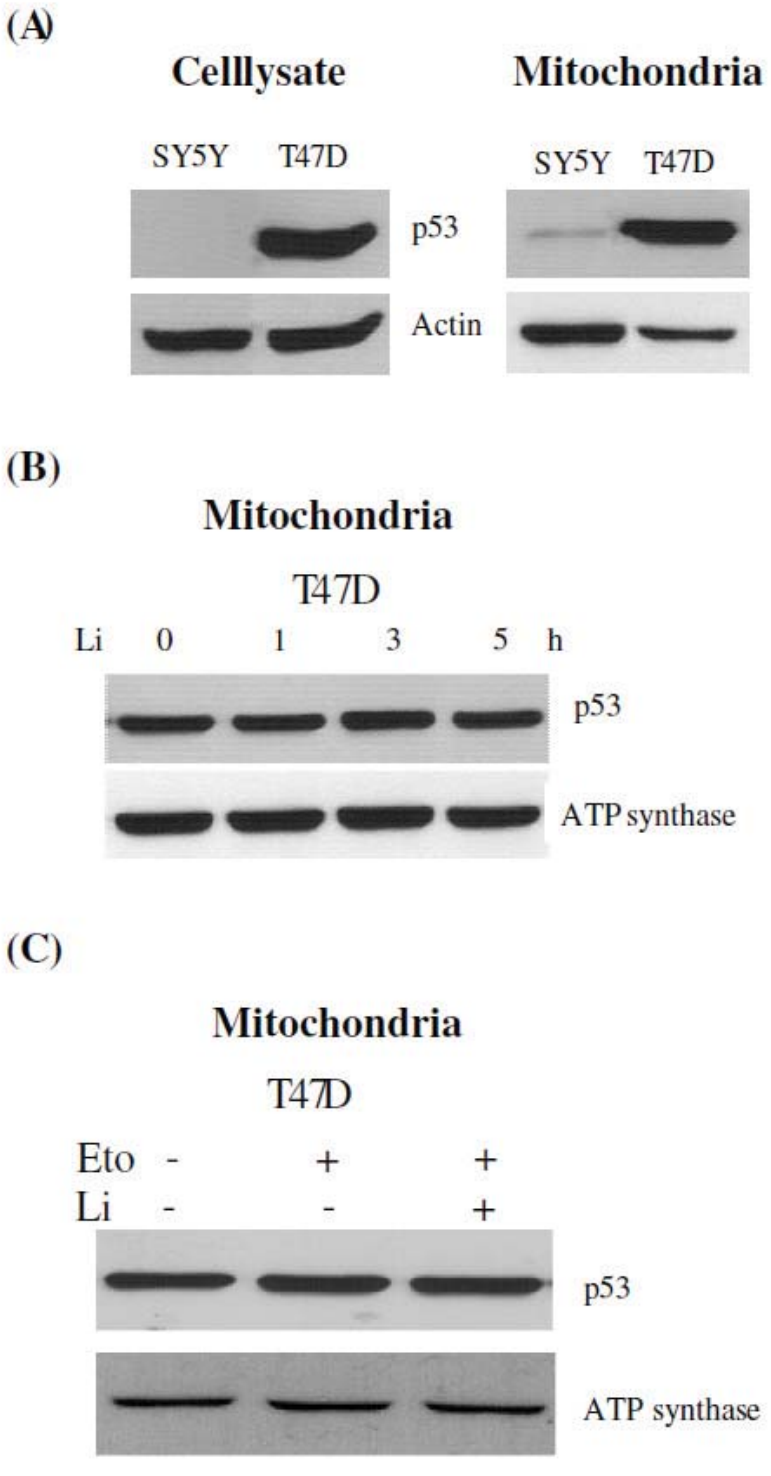
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Figure 4

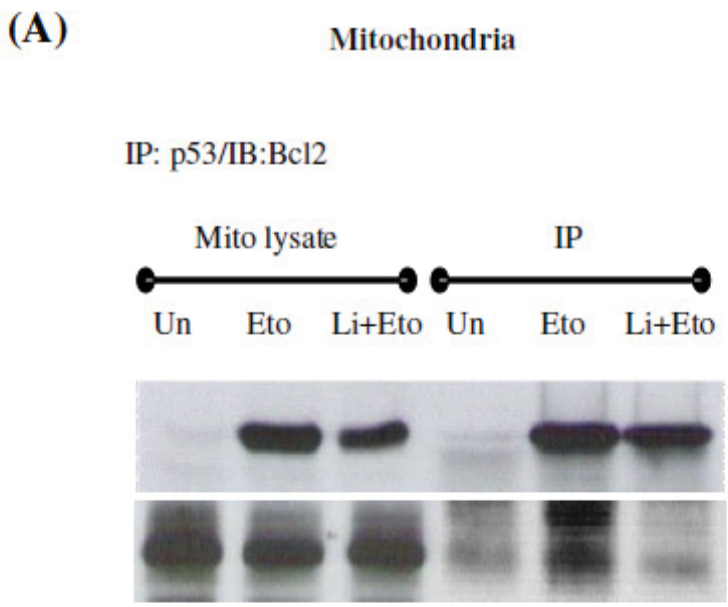
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Figure 5