

Lithium-mediated downregulation of PKB/Akt and cyclin E with growth inhibition in hepatocellular carcinoma cells

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We studied *in vitro* effects of glycogen synthase kinase 3 β (GSK3 β)-inhibitor lithium on the growth of hepatocellular carcinoma (HCC) cells. Lithium induced strong growth inhibition (>70%) in 75% ($n = 9$ of 12) of cell lines, apparently independent from the status of major genes that are mutated in HCC including p53, p16^{INK4a}, β -catenin and Axin1. Comparative studies with a growth-sensitive Huh7 and growth-resistant Hep40 cell lines showed that lithium induces growth arrest in Huh7 cells but not in Hep40 cells. Lithium induced the accumulation of N-terminally phosphorylated inactive form of GSK3 β with concomitant increase in β -catenin and β -catenin/TCF transcriptional activity in both cell lines. This suggests that lithium-mediated HCC growth inhibition is independent of its well-known stimulatory effect on Wnt- β -catenin signaling. The main differences between Huh7 and Hep40 responses to lithium treatment were observed at the levels PKB/Akt and cyclin E proteins. Lithium induced depletion of both proteins in growth-sensitive Huh7, but not in growth-resistant Hep40 cells. PKB/Akt and Cyclin E are 2 major proteins that are known to be constitutively active in HCC. The targeting of both proteins with lithium may be the main reason why most HCC cells are responsive to lithium-mediated growth inhibition, independent of their p53, retinoblastoma and Wnt- β -catenin pathways. The exploration of molecular mechanisms involved in lithium-mediated growth inhibition in relation with PKB/Akt and cyclin E downregulation may provide new insights for therapy of liver tumors.

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Lithium is the main therapeutic agent for the treatment of bipolar disorders, but its mechanism of action is poorly understood.¹ Mechanistic studies have shown that lithium affects profoundly the embryonic development and tissue patterning in various organisms.² The effects of lithium on the embryonic development of *Xenopus* is quite similar to the effects observed upon experimental activation of Wnt- β -catenin pathway.^{2,3–6} This similarity is due to the inhibitory effects of lithium on the activity of glycogen synthase kinase-3 β (GSK-3 β) enzyme,^{7–9} which plays a key role in the regulation of Wnt- β -catenin signaling pathway. In the absence of Wnt signals, GSK-3 β -mediated N-terminal phosphorylation is required for ubiquitin-mediated degradation of β -catenin.^{10,11} Exposure of cells to either Wnt ligands or lithium treatment results in the inhibition of GSK-3 β activity, whereby leading to the cytoplasmic accumulation of β -catenin. Stabilized β -catenin forms nuclear complexes with a family of lymphoid enhancer/T-cell transcription factor (TCF) proteins, leading to the up-regulation of TCF target genes.^{12–15}

The wnt- β -catenin pathway is one of the most commonly activated signaling pathways in different tumor types, including hepatocellular carcinoma (HCC). The aberrant activation of this pathway may occur *via* different mechanisms, but it commonly involves the cytoplasmic or nuclear accumulation of β -catenin protein.^{16–25} Mutational inactivation of the APC gene is the main cause of β -catenin accumulation in >80% of colorectal cancers.²⁶ Oncogenic mutations almost always affecting the N-terminal phosphorylation domain of β -catenin are rare in colorectal cancers,²⁶ but quite common in hepatoblastomas.^{21,27,28} In HCC, β -catenin, Axin1 and p53 mutations are the main sources of β -cat-

enin accumulation.^{29–35} Thus, lithium seems to display an activity (accumulation of β -catenin) that can mimic all known causes of aberrant wnt- β -catenin activation in different types of cancer.

We studied the effects of lithium as an experimental drug for activation of the wnt- β -catenin signaling pathway in HCC cell lines. Our initial studies led us to unexpected finding that the lithium-induced activation of this pathway leads to growth inhibition rather than growth stimulation, independent of the status of wnt- β -catenin pathway genes, in HCC cells. Further studies on selected cell lines showed that lithium is able to activate wnt- β -catenin pathway in HCC cells, but its additional inhibitory effects on PKB/Akt and cyclin E proteins result in a cell cycle arrest, overriding any potential growth stimulatory effect that could be mediated by this pathway. Our studies also provide experimental evidence that lithium or lithium-like drugs are potential candidates for therapy of liver tumors.

Material and methods

Cell lines

The origins and culture conditions of HCC cell lines used here have been reported recently.⁵¹ Lithium treatment was carried out in 12-well plates, 6-well plates or 10-cm culture dishes, depending on the type of experiments. Cells were plated in normal medium for overnight. The following day, the experiments were started by removing the initial medium together with unattached cells, followed by adding fresh medium supplemented with either LiCl or NaCl. Different doses of LiCl (5–30 mM) were used. Unless specified otherwise, negative controls were carried out in medium supplemented with the same concentration of NaCl. For the study of morphological changes, cells were grown on coverslips in 6-well plates, and examined under light microscope. Morphological changes were recorded at different time points by taking digital pictures. For growth assays with different HCC cell lines, 15,000 cells were resuspended in control medium, and plated into 12-well plates. The following day, the medium was removed together with unattached cells, and cells were incubated in a medium containing either 20 mM LiCl or 20 mM NaCl for 4 days. Each experiment was carried out in triplicate and the number of cells were counted manually.

Transfections

Huh7 or Hep40 cells were seeded at a density of 1×10^5 cells/well in a 6-well plate. After 24 hr, transfection was done by using calcium-phosphate method. Two micrograms of pGL3-OT or pGL3-OF reporter plasmids (supplied by Bert Vogelstein) were

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TABLE I - GROWTH RESPONSE OF HEPATOCELLULAR CARCINOMA CELL LINES TO LITHIUM TREATMENT

Cell lines	Retinoblastoma pathway ¹	p53 pathway ¹	Wnt- β -catenin pathway ²	Cell growth (lithium) ³	Cell growth (sodium) ³	Li-induced growth inhibition (%) ⁴
Huh7	Normal	p53 (Y220C)	Normal	0.2 \pm 0.1	17.9 \pm 3.0	98.9
SK-Hep1	p16 ^{INK4a} (HD)	p14 ^{ARF} (HD)	Normal	0.5 \pm 0.2	35.3 \pm 7.4	98.6
SNU-475	p16 ^{INK4a} (PM)	p53(N239D/C275R/N288S)	Axin1 (HD)	0.4 \pm 0.3	8.3 \pm 0.3	95.2
SNU-182	Normal	p53 (S215I)	Normal	0.9 \pm 0.0	8.4 \pm 0.4	89.3
SNU-387	p16 ^{INK4a} (HD)	p53 (L164X)	Normal	0.7 \pm 0.3	5.2 \pm 0.8	86.5
PLC/PRF/5	Normal	p14 ^{ARF} (HD)	Axin1 (Δ E4/-)	2.7 \pm 0.9	18.0 \pm 2.2	85.0
Mahlavu	Normal	p53 (R249S)	Normal	6.7 \pm 1.6	41.3 \pm 5.3	83.8
HepG2	Normal	Normal	β -catenin (Δ E3/wt)	0.5 \pm 0.0	2.5 \pm 0.7	80.0
Hep3B	pRb (undetected)	p53 (HD)	Normal	0.5 \pm 0.2	1.7 \pm 0.3	70.6
SNU-449	p16 ^{INK4a} (HD)	p53 (K139R/A161T)	Normal	4.7 \pm 1.0	10.2 \pm 1.1	53.9
FOCUS	pRb (decreased)	p14 ^{ARF} (HD)	Normal	6.0 \pm 0.0	12.3 \pm 1.2	51.2
Hep40	pRb (undetected)	p53 (undetected)	Axin1 (R454H) ⁵	3.1 \pm 0.8	3.7 \pm 0.8	16.2

¹From Ref.⁵¹.²Data for β -catenin from Ref. 35, data for Axin1 from our study.³Number of cells ($\times 10^4$) \pm SD ($n = 3$) at Day 4 of culture in the presence of 20 mM LiCl or 20 mM NaCl.⁴Based on the comparison with LiCl vs. NaCl treatment.⁵The functional significance of this missense mutation that affects an evolutionarily conserved residue is not known. WT, wild-type; HD, homozygous deletion; PM, promoter hypermethylation.

cotransfected with 0.5 μ g pEGFP-N2 expression vector (Clontech, Palo Alto, CA) to check transfection efficiency. Sixteen hours post-transfection, the medium was replaced with a fresh medium containing 10 mM LiCl, 20 mM LiCl or 20 mM NaCl. After 48 hr of treatment, percentage of transfected cells was calculated by counting GFP-positive and GFP-negative cells, just before performing luciferase assay. Luciferase Reporter Gene Assay, constant light signal kit (Roche Diagnostics GmbH, Mannheim, Germany) was used, as described by the supplier, and luciferase activities were read with the Reporter[®] Microplate Luminometer (Turner BioSystems Inc., Sunnyvale, CA). Arbitrary units of luciferase activity were subtracted from background signal and normalized luciferase units were calculated by taking into account the transfection efficiency of each transfection. All experiments were carried out in triplicates.

Western blotting

Detergent-soluble cell lysates were prepared and used for Western blot analysis, as described previously.⁵¹ Antibodies to β -catenin (Santa Cruz Biotechnology, Santa Cruz, CA), Cyclin D1 (Santa Cruz Biotechnology), Cyclin E (Pharmingen, San Diego, CA), GSK-3 β (Pharmingen, San Diego, CA) and PKB/Akt, phospho-Serine²⁵⁶ FKHR, PTEN, phospho-serine⁹ GSK-3 β in PKB/Akt sampler kit (Cell Signaling Technology, Inc., Beverly, MA), CDK2 (Santa Cruz Biotechnology) CDK4 (Santa Cruz Biotechnology) and calnexin (Sigma, St. Louis, MO) were obtained commercially. Monoclonal antibody to CK18 was kindly provided by D. Bellet (Institut Gustave Roussy, Paris, France). To generate a linear response in western blotting assays, we used appropriate amounts of proteins from cell lysates for SDS-PAGE and an ECL kit (Amersham Life Science, Inc., Piscataway, NJ) for detection of antigen-antibody complexes. Equal protein loading was verified by Western blot assay with CK18 or calnexin. Experiments were repeated several times with different batches of cell lysates for verification.

BrdU labeling and cell cycle analysis

For BrdU incorporation, cells were incubated with 30 μ M BrdU for 4 hr before fixation with ice-cold 70% ethanol for 10 min. After DNA denaturation in 2 N HCl for 20 min, cells were incubated with FITC-conjugated anti-BrdU antibody (DakoCytomation, Glostrup, Denmark), cells were counterstained with Hoechst 33258 (Sigma-Aldrich, Co., Taufkirchen, Germany). Pictures of FITC and Hoechst 33258 fluorescences were taken with different

filter sets and images were merged using Adobe Photoshop 3.05 software.

For cell cycle analysis, asynchronously growing cells were harvested at 48 hr after addition of 20 mM NaCl or 20 mM LiCl. The distributions of cells in different stages of the cell cycle were quantified by flow cytometric cell cycle analysis of DNA content. For DNA profiles, cells were harvested and fixed in 70% ethanol at 4°C. After washing away the ethanol, the cells were incubated in a solution containing 10 μ g/ml DNase-free RNase at 37°C for 30 min, and then stained with 20 μ g/ml propidium iodide solution prepared in 0.5 mM Tris (Sigma-Aldrich, Taufkirchen, Germany), 1.5 mM Spermine tetrahydrochloride (Sigma-Aldrich), 0.1% Nonidet P40 (Sigma-Aldrich) and 3.4 mM Trisodium citrate (Sigma-Aldrich) at room temperature in the dark. At least 15,000 stained cells were acquired using CellQuest software by FACSCalibur cell sorter (Becton Dickinson, Mountainview, CA). Cell cycle distributions were analyzed by using ModFit software and cell cycle profiles were visualized using WinMDI v2.8 software developed by Joseph Trotter (Scripps Research Institute, San Diego, CA; <http://facs.scripps.edu/software.html>).

Tests for apoptosis

Cells were grown up to 72 hr in either LiCl- or NaCl-containing medium, in parallel with normal culture medium. Apoptosis was tested using NAPO and Annexin V assays, as described.³⁷

RT-PCR and Real-Time PCR analyses of cyclin E transcripts

Total RNA was extracted and used for cDNA synthesis and semi-quantitative RT-PCR analysis. The detection of cyclin E and GAPDH mRNA levels were carried out as described previously.⁵¹ The primers were: Cyclin E-F (5'-TTGACCGGTATATGGCGA-CACAAG-3') and Cyclin E-R (5'-ATGATACAAGGCCGAAGC-AGCAAG-3') for the detection of Cyclin E expression; GAPDH -F (5'-GGCTGAGAACGGGAAGCTTGTTCAT-3') and GAPDH -R (5'-CAGCCTTCTCCATGGTGGTGAAGA-3') for the detection of GAPDH expression. The amplification cycle included a denaturation step of 94°C for 5 min, followed by 32 cycles of (for Cyclin E) or 24 cycles of (for GAPDH) 94°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec and concluded with a final primer extension step of 72°C for 5 min.

The iCycler iQ real-time PCR detection system (Bio-Rad, Richmond, CA) was used for real-time PCR of cyclin E and GAPDH. The detection of amplification product was detected with SYBR Green I, using the primer pairs described above.

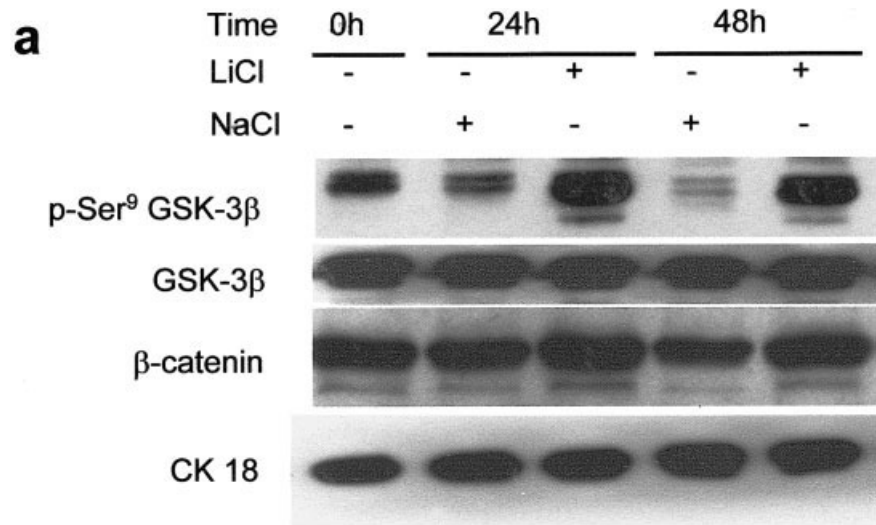
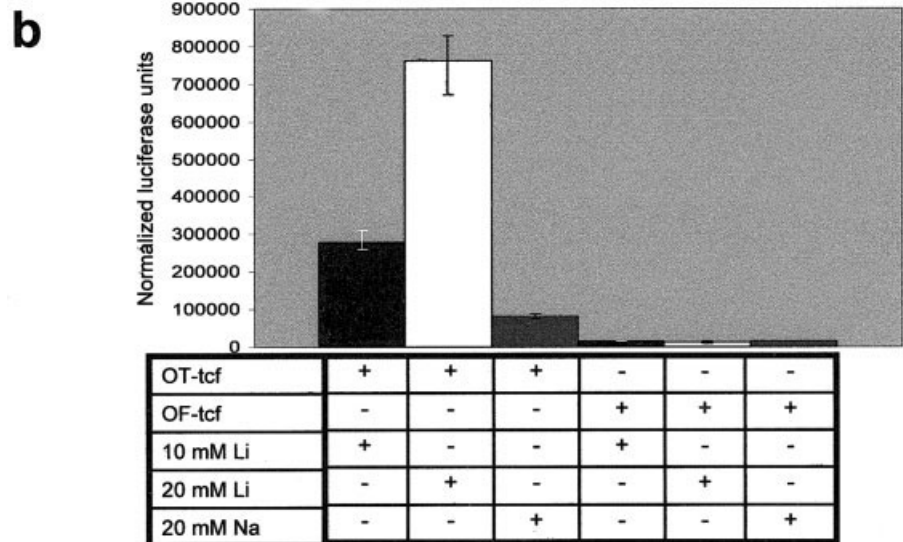


FIGURE 1 – Lithium causes inhibition of GSK-3β, stabilization of β-catenin, and activates β-catenin/TCF reporter pGL3-OT. (a) Huh7 cells were incubated in the presence of either 20 mM LiCl or 20 mM NaCl, and cell lysates were subjected to Western blot assays for total GSK-3β, phospho-serine⁹-GSK-3β, β-catenin and cytokeratin 18 (CK18) at 0, 24 and 48 hr, respectively. LiCl has no effect on total GSK-3β levels, but it causes strong accumulation of phospho-serine⁹-GSK-3β, as well as a weak but reproducible accumulation of β-catenin. CK18 was used as a loading control. (b) Cells were treated with LiCl or NaCl and tested for β-catenin/TCF transcriptional activity using pGL3-OT (OT) reporter plasmid. The pGL3-OF (OF) reporter was used as a negative control. Normalized luciferase activity is the mean ± SD from 3 independent experiments. LiCl causes dose-dependent induction of pGL3-OT reporter activity.



Axin1 mutation analysis

First strand cDNAs of HCC cell lines were prepared by Revert-Aid First Strand cDNA synthesis kit (MBI Fermentas GmbH, St-Leon-Rot, Germany) and screened for possible mutations in a region spanning part of exon 2, exon 3, and 4 and part of 5. This region that encodes amino acids 325–433 (according to GenBank entry: AF009674) is known to be affected by 50% of *Axin1* gene mutations that have been identified in HCC.^{32–34} Cell line cDNAs were amplified and *Axin1* cDNA-positive samples (all except SNU475) were subjected to direct sequencing (Perkin-Elmer Big-Dye Terminal Cycle Sequencing) in both directions using the AxcDNA-Forward (5'-CAACGACAGCGAGCAGCAGCAGA-3) and AxcDNA-Reverse (5'-GAGGCAGCTTGTGACACGGC-3) primer pair. Standard conditions for PCR were 5 pmol/μl each primer, 200 μM dNTPs, 1× PCR buffer containing 1.5 MgCl₂, 1% DMSO and 0.25 U *Taq* DNA polymerase.

Results

Lithium inhibits the growth of most hepatocellular carcinoma cells in p53, p14^{ARF}, p16^{INK4a}, β-catenin and Axin1-independent manner

It is now well established that 2 major components of the Wnt-β-catenin pathway, namely β-catenin and *Axin1* genes are fre-

quent targets for mutation in HCC, suggesting that the constitutive activation of Wnt-β-catenin pathway may play a major role in these cancers. The effects of constitutively activated Wnt-β-catenin pathway, however, are largely unknown in HCC. To study the hepatocellular effects of Wnt-β-catenin signaling, we used an experimental system based on the use of lithium as an inhibitor of GSK-3β enzyme. First, we collected data on the status of *p53*, *p14^{ARF}*, *p16^{INK4a}*, *Retinoblastoma*, *Axin1* and β-catenin genes in a panel of 12 HCC cell lines. As shown in Table I, the status of these genes, except *Axin1*, was already known. *Axin1* gene was analyzed as described in Material and Methods section. In addition to mutations reported previously in SNU-475 and PLC/PRF/5 cell lines, Hep40 cell line displayed a missense mutation in a conserved amino acid residue (R454H) of *Axin1*. There was no detectable *Axin1* mutation in other cell lines listed (Table I). Next, we studied growth response of the panel of HCC cell lines. Equal number of cells were plated from each cell line and cultivated in the presence of either 20 mM LiCl or 20 mM NaCl (control medium) for 4 days, and the number of cells was counted manually. The results of these cell growth experiments were shown in Table I. Of 12 cell lines tested, 9 (75%) responded with a strong growth inhibition ranging between 70–99%. The remaining 3 cell lines were partially resistant to lithium-induced growth inhibition as their growth inhibition was 16–54%. None of the cell lines

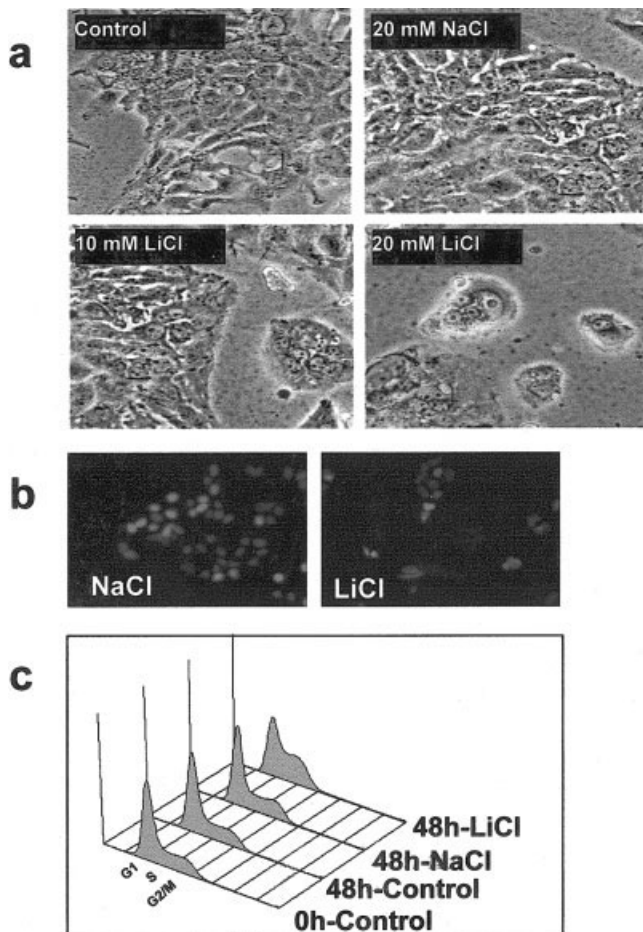


FIGURE 2 – Lithium changes the morphology and inhibits the proliferation of Huh7 cells. (a) Huh7 cells were incubated in the absence (control) or in the presence of either LiCl or NaCl, and light microscope pictures of live cells were taken at 72 hr. Lithium treatment causes tight clustering of cells with scanty cytoplasm some of which have multiple nuclei. This effect is more pronounced with 20 mM LiCl, and no such effect is seen with 20 mM NaCl. (b) Huh7 cells were grown as described in (a) for 44 hr, then incubated in the presence of BrdU for an additional 4 hr, fixed and immunostained for BrdU. Nuclear DNA was counterstained with Hoechst 33258. BrdU-positive and BrdU-negative nuclei stain green and blue, respectively. Note strong inhibition of BrdU incorporation under lithium treatment. (c) Huh7 cells were grown as described in (a) for 48 hr, fixed, DNA-stained with propidium iodide, subjected to flow cytometry, and cell cycles profiles were visualized. The peak of G2/M phase cells decreased, whereas G1/S fraction increased with lithium treatment.

tested responded to lithium treatment by a growth increase (Table I).

These observations showed that lithium was an efficient growth inhibitor of most HCC cell lines. To our surprise, there was no obvious association between the status Wnt- β -catenin pathway genes (β -catenin and *Axin1*) in these cell lines and their response to lithium treatment. Similarly, lithium response also seemed to be independent of *p53* gene and *INK4a/ARF* locus. Most of growth-responsive cell lines (8 of 9) expressed Retinoblastoma (pRb) protein, whereas 2 of 3 growth-resistant cell lines lacked pRb or had decreased expression (Table I).

To further study growth-inhibitory effects of lithium on HCC cell lines, we carried out comparative studies with 2 selected cell lines; Huh7 that responded to lithium by a dramatic growth inhibition (~99%), and Hep40 that was the most growth-resistant cell line displaying only 16% growth inhibition.

Lithium activates Wnt- β -catenin pathway by inhibiting GSK-3 β and inducing β -catenin accumulation in Huh7 hepatocellular carcinoma cells

Lithium activates Wnt- β -catenin signaling as a direct GSK-3 β inhibitor, as well as by inducing the levels of N-terminally (Serine⁹) phosphorylated inactive form of the enzyme.³⁶ Therefore, we first tested the levels of phospho-serine⁹-GSK-3 β by Western blotting. As shown in Figure 1a, the levels of phospho-serine⁹-GSK-3 β displayed a dramatic and sustained increase. In NaCl-treated control cells, there was no accumulation of phospho-serine⁹-GSK-3 β levels, instead there was a progressive decrease, most probably due to cell proliferation. Lithium not only inhibited the decrease of phospho-serine⁹-GSK-3 β levels that occurs in proliferating cells, but also caused a strong accumulation of this inactive form. As the total levels of GSK-3 β did not change under these conditions (Fig. 1a), it is highly likely that the accumulation of phosphorylated/inactive form of GSK-3 β caused a depletion of the nonphosphorylated/active form of the enzyme. We then tested whether lithium-induced inactivation of GSK-3 β affected β -catenin protein levels. As shown in Figure 1a, there was a weak but sustained accumulation of β -catenin protein levels, despite its relatively high basal levels.

As β -catenin binds to TCF/Lef transcription factors to activate Wnt-responsive genes, we evaluated the ability of LiCl to activate Wnt-dependent transcription using TCF/Lef reporter plasmid pGL3-OT.¹² Huh7 cells were treated with 10 or 20 mM LiCl up to 48 hr, and β -catenin/TCF transcriptional activity was tested. NaCl treatment was used as a negative control. As shown in Figure 1b, Huh7 cells displayed a low, but detectable pGL3-OT reporter activity in the absence of lithium treatment, suggesting that β -catenin/TCF transcriptional activity is weakly positive in this cell line. The treatment with 10 mM and 20 mM LiCl resulted in 2.5- and 10-fold increases of pGL3-OT activity, respectively. The pGL3-OF reporter (containing mutant-binding sites) displayed only background activity, with no change with lithium treatment, as expected.

These results demonstrated that lithium causes the accumulation of phosphorylated/inactive forms of GSK-3 β in Huh7 cells, with a concomitant accumulation of β -catenin levels, as well as an increase in β -catenin/TCF reporter activity, similarly to its previously reported effects in 293T kidney epithelial cells.³⁶

Lithium affects morphology and proliferation of Huh7 cells

We studied the effects of lithium treatment on the morphology and growth properties of Huh7 cells. These cells responded to lithium treatment with the formation of tightly clustered cells (Fig. 2a). At 20 mM of lithium treatment, there was an increase in nucleus/cytoplasm ratios, sometimes accompanied with multinuclear cell formation. These morphological changes were specific to lithium, as NaCl-treated cells displayed no morphological changes (Fig. 2a). Representative pictures of cells shown in Figure 2a were taken at 72 hr. The findings were similar at 48 hr (data not shown). Lithium-induced morphological changes were not associated with programmed cell death, at least for 72 hr. Huh7 cells display characteristic features of apoptosis (nuclear condensation, membrane blobbing) as reported previously,³⁷ which are easily distinguished from the morphology shown in Figure 2a. In addition, we carried out apoptosis tests in these cell line under lithium-treatment, using 2 independent tests (NAPO assay³⁷ and Annexin V staining). There was no significant change in the number of apoptotic figures under lithium-treatment, as compared to control and NaCl-containing cultures that remained low (<1%) during 72 hr of culture (data not shown).

To test whether lithium affects the growth of Huh7 cells by inhibiting their proliferation, first, we studied DNA synthesis by BrdU incorporation assay. Huh7 cells were incubated in the presence of either 20 mM LiCl or 20 mM NaCl up to 48 hr. At time 44 hr, BrdU was added into culture medium, and cells were incubated for an additional 4 hr in the presence of BrdU. The immunofluorescence staining of fixed cells for BrdU indicated that DNA

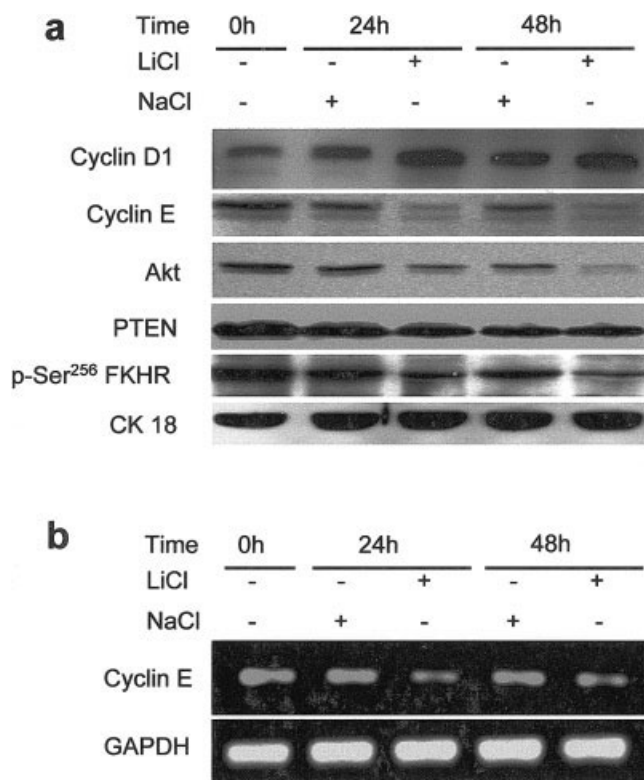


FIGURE 3 – Lithium induces the accumulation of cyclin D1, but provokes a depletion of cyclin E and PKB/Akt proteins in Huh7 cells. (a) Cells were treated with LiCl or NaCl (20 mM each) for 48 hr, and protein lysates, collected at 0 hr, 24 hr and 48 hr, were subjected to Western blot assays for cyclin D1, cyclin E, PKB/Akt, PTEN, phospho-serine²⁵⁶ FKHR and CK18. Note the accumulation of cyclin D1, but depletion of Cyclin E and PKB/Akt levels under lithium treatment. As an indication of PKB/AKT inhibition by lithium treatment, phospho-serine²⁵⁶ FKHR levels also decreased. PTEN levels did not change. CK18 was used as a loading control. (b) Cyclin E and GAPDH transcripts were tested by RT-PCR analysis of total RNA extracted from LiCl- and NaCl-treated Huh7 cells at 0 hr, 24 hr and 48 hr. Lithium caused a decrease in Cyclin E mRNA levels, in parallel to the depletion of its protein levels shown in (a). Real-time PCR analysis of Cyclin E mRNA indicated a 16-fold decrease under lithium treatment.

synthesis was severely impaired after 48 hr of lithium treatment. The degree of DNA synthesis inhibition was quantified by manual counting of BrdU-positive cells. After 4 hr of continuous labeling with BrdU, 33% \pm 4 ($n = 3$) of NaCl-treated control cells displayed positive staining. In contrast, 4 hr of continuous labeling with BrdU yielded only 15% \pm 2 ($n = 3$) positivity with lithium-treated cells. Representative pictures of BrdU experiments were shown in Figure 2b. Flow cytometric analysis of distribution of cells with different DNA contents suggested that lithium-treated cells accumulate at G1/S phase of the cell cycle. Although an accurate calculation of ratios of cells at G1, S and G2/M phases was not possible due to heterogeneous DNA content and lithium-induced clustering of cells, representative data shown in Figure 2c shows that cells at G2/M phase shift toward G1/S peak under LiCl treatment, but not with NaCl treatment. These observations, combined with BrdU experiment that shows strong inhibition of DNA synthesis, are in favor of a lithium-induced G1/S cell cycle arrest.

Lithium induces cyclin E depletion in growth-sensitive Huh7, but not in growth-resistant Hep40 cells

Cyclin D1 and cyclin E are involved in the control of G1 phase of the cell cycle. In addition, cyclin D1 is known to be

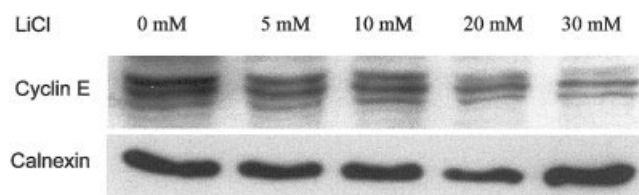


FIGURE 4 – Lithium effect is dose-dependent. Huh7 cells were treated with gradually increasing concentrations of LiCl (0–30 mM) for 48 hr, and protein lysates were subjected to Western blot assay for cyclin E, followed by calnexin as an equal loading control. Note the dose-dependent depletion of Cyclin E under lithium treatment.

upregulated by Wnt- β -catenin signaling.¹⁴ We first tested protein levels of these G1 cyclins in lithium-treated Huh7 cells. As shown in Figure 3a, lithium-treated Huh7 cells displayed a moderate increase in cyclin D1 levels. More interestingly, lithium treatment caused a dramatic fall in the levels of cyclin E protein in these cells that was detected at both 24 hr and 48 hr after lithium treatment (Fig. 3a). There was no change in the levels of cyclin-dependent kinase 4 (CDK4) and CDK2 levels under these conditions (data not shown). To test whether the progressive decrease of cyclin E protein levels was due to a decrease in cyclin E mRNA levels, we carried out RT-PCR and quantitative PCR analyses. As shown in Figure 3b, cyclin E transcript levels also decreased with lithium treatment. The degree of cyclin E transcript downregulation was calculated by additional experiments using real-time PCR. Progressively diluted cDNA preparations obtained from NaCl-treated cells were subjected to real-time PCR, in comparison with samples from LiCl-treated cells. Undiluted samples from LiCl-treated cells provided a signal that was equal to that of a 16-fold diluted samples from NaCl-treated cells (data not shown). This provided evidence for at least 15-fold inhibition of cyclin E mRNA levels by lithium in Huh7 cells.

To test whether the effects of lithium were dose-dependent, Huh7 cells were incubated for 48 hr, in medium supplemented with 5, 10, 20 or 30 mM LiCl, in comparison with normal medium. As shown in Figure 4, cyclin E levels decreased progressively, as the levels of LiCl were raised from 0–30 mM.

These studies indicated that lithium is able to cause a depletion of cyclin E protein in Huh7 cells, and this effect is due, at least in part, to a dramatic fall in cyclin E transcripts, probably because of a transcriptional inhibition, as cyclin E transcripts are better known for transcriptional regulation.³⁸

As cyclin E is required for the transition of cells through G1 to S phase, inhibition of cyclin E expression is probably responsible, at least partially, from lithium-induced growth inhibition of Huh7 cells, and most probably other HCC cell lines that responded to lithium treatment (Table I). If our hypothesis is correct, growth-resistant Hep40 cells should respond to lithium treatment differently. We carried out similar studies in Hep40 cells. First, we tested whether lithium was able to activate wnt- β -catenin signaling in Hep40 cells using β -catenin/TCF reporter assay. As shown in Figure 5a, lithium was able to induce pGL3-OT reporter activity >6-fold with 20 mM in Hep40 cell line. Transcriptional activation of β -catenin/TCF in Hep40 cells was accompanied with accumulation of phospho-serine⁹-GSK-3 β , suggesting that the effects of lithium on wnt- β -catenin signaling were comparable between Hep40 and Huh7 cell lines. Lithium treatment had no effect on cyclin E protein levels in Hep40 cells, unlike Huh7 cells (Fig. 5b). One of the main differences between growth-responsive Huh7 and growth-resistant Hep40 cells was at the level of cyclin E expression. This suggests that lithium is able to inhibit the growth of some HCC cells such as Huh7 cells by interfering with the expression of cyclin E, a critical protein involved in the transition from G1 to S phase of the cell cycle.

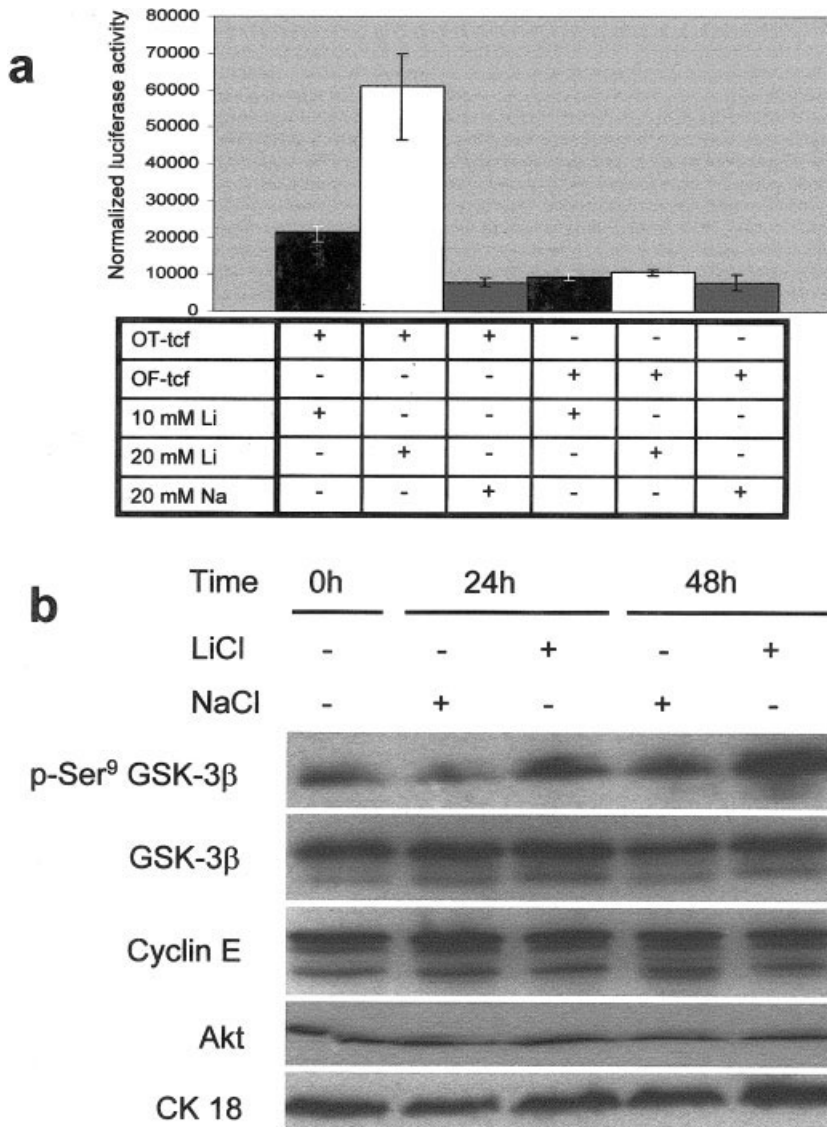


FIGURE 5 – Lithium activates Wnt- β -catenin signaling, but has no effect on cyclin E and PKB/Akt proteins in growth-resistant Hep40 cells. (a) After incubation in the presence of either 20 mM LiCl or 20mM NaCl, Hep40 cell lysates were subjected to reporter assay for β -catenin/TCF-mediated transcription, as described in Figure 1b. The pGL3-OT luciferase activity was induced in the presence of LiCl in a dose-dependent manner. (b) Cells were treated with LiCl or NaCl (20 mM each) for 48 hr, and protein lysates, collected at 0 hr, 24 hr and 48 hr, were subjected to Western blot assays for phospho-serine⁹-GSK-3 β , total GSK-3 β , cyclin E, PKB/Akt and CK18. Lithium caused accumulation of phospho-serine⁹-GSK-3 β , but it had no effect on total GSK-3 β , cyclin E and PKB/Akt levels. CK18 was used as a loading control.

Lithium depletes PKB/Akt in Huh7, but not in Hep40 cells

The molecular mechanisms of lithium-mediated inhibition of cyclin E expression are presently unknown, but this could be due to the inhibition of an upstream signaling event by lithium. PI3K/Akt pathway has been implicated recently in control of HCC cell proliferation.³⁹ Therefore, we also tested protein levels of PKB, also called Akt (PKB/Akt) protein in Huh7 and Hep40 cells. Lithium caused a depletion of PKB/Akt protein levels in Huh7 (Fig. 3a), but not in Hep40 (Fig. 5b) cells. Lithium-mediated depletion of PKB/Akt protein levels was accompanied with a decrease in the phosphorylation of its substrate FKHR⁴⁰ in Huh7 cells, providing indirect evidence that PKB/Akt is constitutively active in this cell line, but its activity is inhibited by lithium treatment. The protein PTEN that acts as an inhibitor of PI3K/Akt signaling⁴¹ did not show detectable change under these conditions (Fig. 3a). Taken together, these observations provide evidence that the inhibition of PKB/Akt or cyclin E expression is implicated in lithium-induced growth control of HCC cells.

Discussion

Our studies on hepatocellular response to lithium show several interesting novel findings. First, HCC cell lines are in general

responsive to lithium treatment. As exemplified by results presented for Huh7 and Hep40 cells, lithium induces the accumulation of inactive phospho-serine⁹ GSK-3 β with concomitant activation of Wnt- β -catenin signaling. Most strikingly, lithium was able to inhibit strongly (>70% inhibition) the growth of 9/12 cell lines tested. This growth-inhibitory response did not require functional *p53*, *p16^{INK4a}*, *p14^{ARF}*, *β -catenin* or *Axin1* genes. This particular growth inhibitory response was accompanied with a depletion of both cyclin E and PKB/Akt proteins in Huh7, a growth-sensitive cell line, whereas there was no such effect in Hep40, a growth-resistant cell line. Cyclin E depletion is apparently regulated at the transcriptional level as we detected 16-fold decrease of cyclin E transcripts under lithium treatment. This defect in cyclin E expression is likely to play the major role in growth inhibition, as Huh7 cells underwent DNA synthesis inhibition as well as an accumulation at the G1/S phase of the cell cycle. This is not unexpected, as cyclin E together with cdk2 is required for the entry of cells from G1 to S phase. Cyclin E is one of the key genes in hepatocellular carcinogenesis, as its upregulation is frequent⁴² and its activation may be closely related to the histopathologic grade and progression of HCC.⁴³ Moreover, suppression of cyclin E overexpression in HCC by RNAi leads to inhibition of DNA synthesis and tumor growth *in vivo*.⁴⁴ The other protein targeted by lithium, PKB/Akt is one of the major kinases that inacti-

vates GSK3 β by N-terminal phosphorylation, although additional kinases have also been implicated.⁴⁵ Thus, before discussing the implications of lithium-mediated PKB/Akt inactivation, accumulation of N-terminally phosphorylated GSK3 β in Huh7, under the same conditions deserves further consideration. It has been reported recently that lithium induces GSK3 β N-terminal phosphorylation through direct inhibition of GSK3 β itself. This lithium-induced N-terminal phosphorylation is based on a mechanism regulating GSK3 β -dependent protein phosphatase 1-inhibitor 2 complex that does not require PKB/Akt.³⁶ Huh7 cells may use such a mechanism to induce N-terminally phosphorylated inactive GSK3 β , upon exposure to lithium, despite the inactivation of PKB/Akt protein.

Inactivation of GSK3 β and activation of wnt- β -catenin signaling is probably one of the mechanisms that explain moderate increase in the levels of cyclin D1 in both Huh7 and Hep40 cells, as this cyclin D1 gene is a transcriptional target for wnt- β -catenin signaling,¹⁴ and GSK-3 β triggers cyclin D1 degradation by phosphorylation.⁴⁶ Lithium-mediated increase in cyclin D1 levels was not sufficient, however, to stimulate cell proliferation of the 2 cell lines studied. Indeed, the data with Huh7 strongly suggests that this wnt- β -catenin-related effect of lithium is override by a concomitant drop in cyclin E levels due to a loss of cyclin E transcripts, which leads to a cell cycle arrest, instead of cell proliferation.

The mechanisms by which cyclin E transcripts are downregulated by lithium treatment in Huh7 cells are presently unknown, but it could be related to downregulation of PKB/Akt function in these cells. Lack of both PKB/Akt and cyclin E downregulation in growth-resistant Hep40 cells under the same conditions further supports this hypothesis, although a direct proof remains missing. PKB/Akt, a kinase with multiple cellular functions, including metabolic control and cell survival, is a critical component of PI3K/Akt pathway that is deregulated in different cancer types.⁴⁷ Recently, PI3K/PKB/Akt signaling acting *via* inhibition of C/EBP α has been described as a molecular mechanism for the development of liver tumors.³⁹ The studies by Wang *et al.*³⁹ clearly establish that PKB/Akt is constitutively active in HCC cells and this activation blocks the growth inhibitory activity of C/EBP α through the PP2A-mediated dephosphorylation. C/EBP α blocks proliferation of hepatocytes by 2 distinct mechanisms;

inhibition of CDK4/CDK2 and repression of E2F.^{48,49} Accordingly, inhibition of PKB/Akt by RNA interference, or by PI3K-inhibitor Wortmannin treatment induces growth arrest in HCC cells. The effects that we observed with Huh7 cells parallel with these observations in terms of lithium-mediated downregulation of PKB/Akt and growth arrest. In addition, we showed that Wortmannin significantly inhibits the growth of Huh7 cells (data not shown). The decrease of cyclin E transcripts upon PKB/Akt depletion in these cells is probably related to the inhibitory effects of C/EBP α on CDK4 and E2F activities. In confirmation of studies reported by Wang *et al.*³⁹ our preliminary studies show that both Huh7 and Hep40 express C/EBP α (data not shown). Interestingly, retinoblastoma protein that is required for C/EBP α -mediated E2F repression is expressed in Huh7, but not in Hep40 cells (Table I).

The activity of PKB/Akt is known to be regulated by its PI3K-dependent phosphorylation.⁴⁷ Regulation by the control of PKB/Akt protein levels has also been described as a novel mechanism.⁵⁰ Interestingly, mTOR pathway seems to be involved in this process, as inhibition of mTOR by rapamycin induces PKB/Akt degradation in endothelial cells.⁵⁰ Presently, it is not known, whether lithium utilizes a similar mechanism to decrease PKB/Akt protein levels in Huh7 cells.

In conclusion, our observations provide evidence for hereto unknown effects of lithium; the downregulation of levels of PKB/Akt and cyclin E protein. These 2 proteins are required for cell survival and proliferation. These newly described effects may count, as least partially, for the ability of lithium to prevent the growth of many HCC cells, apparently independent from the mutational status of major HCC genes such as *p53*, *p16^{INK4a}* and *β -catenin* (Table I). The exploration of molecular mechanisms involved in lithium-mediated HCC growth inhibition in relation with PKB/Akt/cyclin E downregulation may provide new insights for therapy of liver tumors.

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