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Lithium and inhibition of GSK3 β as a potential therapy for serous ovarian cancer

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Abstract

Objective—Lithium chloride has been shown to demonstrate anti-cancer properties at supratherapeutic doses. This study was designed to determine whether lithium chloride, as a single agent or in combination with cytotoxic agents reduces ovarian cancer cell growth and metabolic activity at clinically achievable levels.

Materials and Methods—We studied the effects of lithium chloride on two high-grade serous ovarian cancer cell lines, SKOV3 and OVCA 433, and primary cultures developed from ascitic fluid collected from patients with metastatic high-grade serous ovarian cancer. We assessed proliferation and metabolism using cell cycle analysis, MTT assays and cellular proliferation and clonogenic potential assays.

Results—Treatment with 1mM LiCl had no effect on the cell cycle distribution or metabolic activity of the SKOV3 and OVCA 433 cell lines. Combination treatment with cisplatin or paclitaxel led to statistically significant decreases in metabolic activity in the OVCA 433 cell line and 50% of cultures investigated. The decreased metabolic activity was not, however, associated with decreased cell growth or clonogenic potential.

Conclusions—Combination treatment with LiCl and cytotoxic agents at physiologically achievable drug concentrations reduces ovarian cancer cell metabolism but does not appear to effect cellular proliferation. The potential for combined lithium/cytotoxic therapies appears to be limited based on our analysis of both established cell lines and short term ovarian cancer cultures.

Introduction

Ovarian cancer is the second most common gynecologic cancer in the United States and the most frequent cause of death among these malignancies. In 2012, it is estimated that 22,280 new cases will be diagnosed and 15,500 women will die of the disease.[1] Most women present with advanced stage disease and are treated with surgery followed by adjuvant chemotherapy utilizing a taxane/platinum based regimen. Despite improvements in chemotherapy administration, 80-85% of women with advanced stage ovarian cancer eventually recur and die of their disease due to the development of drug resistance.[2] There is urgent need for novel and more effective treatments for ovarian cancer.

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Conflict of Interest Statement

The authors report no conflicts of interest.

Glycogen synthase kinase 3 β (GSK3 β) is a serine/threonine protein kinase and plays an important role in cellular metabolism, transcription, cell cycle division, apoptosis and maintenance of stem cells.[3] Biological pathways involving GSK3 β have been implicated in diabetes and Alzheimer's disease. Its role in tumorigenesis is complex. As part of the canonical Wnt pathway, phosphorylation of β -catenin by GSK3 β leads to growth arrest.[4] Conversely, GSK3 β has been shown to activate NF κ B dependent gene transcription leading to cellular proliferation and survival.[5] GSK3 β has been demonstrated to be involved in tumorigenesis in gliomas, pancreatic, colorectal and prostate cancers.[5-9]

Lithium chloride (LiCl) inhibits GSK3 β through two mechanisms. It competes with magnesium to bind to GSK3 β and disrupts its catalytic function due to its lower charge density.[10,11] Additionally, LiCl is associated with phosphorylation of a regulatory serine-9 on the N-terminal region, which is a principal regulator of GSK3 β . [12] *In vitro* studies have demonstrated that treatment with lithium chloride can inhibit growth of endometrial cancers,[13] gliomas,[14] carcinoid tumors[15] and colorectal cancers [16] and ovarian cancers.[17]

Therapeutic serum levels for lithium chloride are 0.8-1.2meq/L (0.8-1.2mM) with lithium toxicity usually beginning at 1.5-2meq/L (1.5-2mM).[18] Prior *in vitro* work investigating the effect of LiCl in tumorigenesis utilized supraphysiologic doses limiting applicability to *in vivo* models.[17] Our study assessed the effects of a physiologic dose of LiCl on cellular metabolism and proliferation of ovarian cancer cells.

Materials and Methods

Cell lines and culture conditions

The SKOV3 and OVCA 433 serous ovarian cancer cell lines were a gift from Dr. David Curiel, Department of Radiation Oncology, Washington University School of Medicine. Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic/antimycotic.

Primary ovarian cancer cells from ascites

All patient specimens were collected under Washington University Institutional Review Board approved protocols. We established short term cultures of high-grade serous ovarian cancer ascites as described by Sonnemann et al.[19] Briefly, 50-100ml of ascites from patients with metastatic, high-grade serous ovarian cancer was collected at the time of surgery and centrifuged at 3000rpm at room temperature. The cellular pellet was washed twice with PBS and then resuspended in DMEM/F12 supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic/antimycotic and plated in T75 (TPP #90076, 75cm² surface area) flasks. Cells were passaged when they became 70-80% confluent. All analyses were performed on passages 2 through 5.

Cell cycle analysis

SKOV3 and OVCA 433 cells were seeded in 10cm dishes (TPP #93100, 60cm² surface area) at a starting concentration of 1x10⁶ cells per plate. Cells were treated with LiCl, cisplatin and/or paclitaxel 24 hours after initial plating. After a 96 hour treatment course the cells were trypsinized and washed twice with PBS. The cellular pellet was resuspended in 100% ethanol and incubated at -20°C overnight. The cells were washed with PBS supplemented with 1% FBS and then resuspended in fresh nuclei staining buffer (250ug/ml RNase A and 10ug/ml propidium iodide). Flow cytometry was performed using a BD FACSCalibur flow cytometer and cell cycle histograms were generated after analysis using FlowJo (v7.6.5, Tree Star, Inc, Ashland, OR).

MTT assays

SKOV3 and OVCA 433 cells were seeded at a density of 1×10^3 cells per well in a 96 well plate (TPP #92096, 0.33cm^2 surface area). Cells were treated with LiCl, cisplatin, paclitaxel or a combination of LiCl with either cisplatin or paclitaxel and allowed to grow undisturbed for 96 hours at which point the growth media was replaced with fresh media containing 15% MTT (5mg/ml; Sigma-Aldrich) and incubated for 3.5 hours at 37°C . Cells were lysed using a solution of 4mM HCl and 0.1% NP40 in isopropanol. Absorbance was measured on a plate reader at 595nm.

Cell growth and colony formation assays

Cells were seeded at a density of 2×10^4 cells per well in a 6 well plate (TPP #92006 9cm^2 surface area) and treated 24 hours after plating with LiCl, cisplatin, paclitaxel or a combination of LiCl with either cisplatin or paclitaxel. Following a 96-hour treatment course the cells were washed with PBS, trypsinized and counted using the trypan blue exclusion method.

For the colony formation assay, 300 cells from each treatment group were plated in 10cm dishes. After fifteen days the growth media was aspirated and the cells were stained with methylene blue and incubated at room temperature for 1 hour. Colonies were counted and plates photographed.

Statistical considerations

Cell cycle analyses, MTT assays, cell growth and colony formation assays were performed in at least triplicate. Means from all experiments were combined and normalized to the means of the untreated cells and compared using the Wilcoxon Rank-sum test. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using Stata v9.2, (StataCorp LP, College Station, TX).

Results

Cell cycle analysis

Ninety-six hour treatment with 10mM LiCl was associated with significant changes in the cell cycle distribution of both the OVCA 433 and SKOV3 cell lines (Figure 1A). The percentage of cells in G2/M phase increased 68% and 36%, respectively ($p < 0.001$ for both cell lines, Wilcoxon Rank-sum test) (Figure 1B). The increase in G2/M was associated with 10% and 22% decreases in the numbers of cells in G1 phase in the OVCA 433 and SKOV3 cell lines ($p = 0.04$ and 0.01 , respectively). No change in the fraction of cells in the G1, S or G2/M phase was seen when the cells were treated with 1mM LiCl (a physiologically achievable concentration).

LiCl effects on cellular metabolic activity

Metabolic activity was significantly reduced in the OVCA 433 and SKOV3 cell lines after treatment for 96 hours with 10mM LiCl (83% and 63% reduction, respectively) as measured by MTT assays (Figure 2A). The effect was evident as early as 48 hours after treatment and showed clear cell density effects, with the greatest inhibition seen with rapidly proliferating, subconfluent cultures (data not shown). Treatment with 1mM LiCl resulted in no change in metabolic activity in either cell line.

Although 1mM LiCl alone did not affect cell cycle (FACS analysis) or cellular metabolic activity (MTT assays), we hypothesized that low dose lithium might sensitize ovarian cancer cells to cytotoxic agents. MTT analyses investigating the growth inhibitory effects of

cisplatin (40nM-20μM) and paclitaxel (0.4-100nM) established 1μM cisplatin and 2nM paclitaxel as doses that inhibited ovarian cancer cell lines by approximately 50% (dose response data not shown). Metabolic activity of the OVCA433 and SKOV3 cell lines decreased by 48% and 49% with 1μM cisplatin and by 48% and 31% with 2nM paclitaxel treatment, respectively. Treatment with 1mM LiCl alone demonstrated no significant decrease in either cell line ($p=0.38$ and $p=0.32$, respectively). In the OVCA 433 cell line, combination treatment with LiCl and cisplatin or paclitaxel resulted in additional 14% and 20% decreases over treatment with either agent alone in the OVCA 433 cell line ($p=0.06$ and $p=0.04$, respectively). No similar decrease was seen in the SKOV3 cell line (Figure 2A).

LiCl effects in primary ovarian cancer cultures

Given the combined activity of LiCl with cisplatin and paclitaxel demonstrated in the OVCA 433 cell line, we assessed the effects of combination treatment with LiCl and cytotoxics in ovarian cancer cells isolated from ascitic fluid grown in primary culture. Primary cultures were derived from the ascites of six patients with stage III high-grade serous ovarian cancer.

Treatment with 10mM LiCl led to 21-60% decreases in the measured metabolic activity (Figure 3), an effect size slightly smaller than that seen with the established cell lines. Of the six primary cultures one (WUOVc6) demonstrated a modest, but significant decrease with 1mM LiCl treatment (Figure 3). Treatment with 1μM cisplatin or 2nM paclitaxel alone resulted in decreases in metabolic activity of 43-77% and 49-81%, respectively.

Overall, three of the six primary cultures (WUOVc1-3) showed a significant combinatorial effect with 1mM LiCl and either cisplatin or paclitaxel with an additional 5-8% absolute decrease in their relative metabolic activity from treatment with a single agent. The remaining three primary cultures (WUOVc4-6) displayed no combinatorial effect (Figure 3).

LiCl effects on cellular proliferation and clonogenic potential

The combinatorial effect of LiCl and cytotoxic agents was assessed in the OVCA 433 cell line. Cell counts after a 96 hour treatment course demonstrated no differences between untreated cells and those treated with 1mM LiCl ($p=0.98$). Similarly, no additional decrease in cellular growth was seen in the cells treated with combination LiCl and cisplatin or paclitaxel ($p=0.15$ and 0.45 , respectively) compared to each individual cytotoxic agent (Figure 4A). LiCl alone, or in combination with cisplatin or paclitaxel, had no effect on the SKOV3 cell line (data not shown).

Lithium chloride, as a single agent or in combination with cytotoxics had limited effect on clonogenic potential (Figure 4B). OVCA 433 cells were harvested after the 96 hour exposure to drug and plated in growth media and allowed to grow colonies for fifteen days without any additional treatment. After staining, colonies containing more than fifty cells were counted (Figure 4B, upper panel). Whereas cells previously treated with cisplatin and paclitaxel, to a lesser extent, demonstrated a significant decrease in their clonogenic potential prior treatment with 1mM LiCl demonstrated only a 12% decrease in the total number of colonies compared to untreated cells ($p=0.04$). No significant difference was seen between the cisplatin/paclitaxel groups and their LiCl combination groups ($p=0.58$ and $p=0.69$, respectively) (Figure 4B, lower panel).

A small, but significant decrease in the clonogenic potential of OVCA 433 cells treated with 1mM LiCl alone was evident. Despite a decrease in the number of colonies on the 1mM LiCl plates, the colonies were larger and contained more cells as compared to the untreated plate (Figure 4B, upper panel).

Discussion

We assessed the effects of LiCl on cell cycle, metabolism, proliferation and clonogenic potential in two high-grade serous ovarian cancer cell lines. Treatment with a supraphysiologic dose of LiCl led to perturbations of cell cycle and cellular metabolism similar to the results demonstrated by Cao *et al.*[17] In our experiments, however, the increase in the percentage cells in G2/M phase came as a result of a decrease in the percentage of cells in G1 phase.

A pharmacologically achievable drug level (1mM LiCl) had no effects on cell cycle or cellular metabolism. However, combination treatment with 1mM LiCl and either cisplatin or paclitaxel resulted in decreased metabolic activity in the OVCA 433 cell line when compared to treatment with either cytotoxic alone as assessed by MTT assays. Similarly, 50% of the primary cultures derived from ascites demonstrated a combinatorial effect.

The results of our cell proliferation assays in the OVCA 433 cell line is in concert with the results of the MTT assays with a significant decrease seen in the number of cancer cells after treatment with cisplatin or paclitaxel. However, we did not find a combined effect between LiCl and either cisplatin or paclitaxel. While we did not perform cellular proliferation and clonogenic assays with the primary cultures, we hypothesize that the results would be similar to those demonstrated in the primary cell lines and would not demonstrate significant effects on cellular proliferation, either alone or in combination with other cytotoxic agents.

The combinatorial effects identified by the MTT assays reflect a reduction in metabolic activity, a relative reduction in cell number or a combination of these two effects. As no decrease in cell proliferation or clonogenic potential was demonstrated, we hypothesize that lithium's effect, if any, is limited to a disruption of cellular metabolism.

While treatment with lithium chloride has been shown to be effective by *in vitro* studies in multiple cancer types, *in vivo* studies are limited. A recent phase II trial of LiCl in low-grade neuroendocrine tumors failed to demonstrate a clinical response.[20] These disparate results may be due to the supraphysiologic doses used in the pre-clinical studies. While our study is limited by its *in vitro* nature it suggests that lithium chloride, at physiologic levels, would be of limited clinical benefit in the treatment of ovarian cancer.

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Figure 1.

Effects of LiCl on cell cycle **A.** Representative DNA content histograms for OVCA 433 and SKOV3 cells after 96 hours treatment with 1 and 10mM LiCl. **B.** Increase in cells in G2/M phase with concomitant reduction in G1 fraction with 10mM LiCl. The data represents the mean of at least four replicates of each experiment and the standard error of the mean (SEM) compared using the Wilcoxon Rank-sum test.

Figure 2.

Evaluation of metabolic activity in established serous ovarian cancer cell lines demonstrated reduced metabolic activity with combined lithium/cytotoxic treatment in OVCA 433 and no effect in SKOV3. All values are normalized to the untreated control and data is presented as the mean of four replicates of each experiment and the SEM compared using the Wilcoxon Rank-sum test.

Figure 3.

Metabolic activity was evaluated in six primary ovarian cancer cell cultures. Three of the cultures (WUOVCa 1, 3, 4) were responsive to combination treatment with LiCl and cytotoxics. The remaining cultures (WUOVCa 2, 5, 6) demonstrated no response to combination treatment. All values are normalized to the untreated control and data is presented as the mean of four replicates of each experiment and the SEM compared using the Wilcoxon Rank-sum test.

Figure 4.

LiCl effects on OVCA 433 cell proliferation and clonogenic potential. **A.** Effects on cell number after 96 hours treatment. All values normalized to the untreated control of experiments. **B.** Colony formation 15 days post-treatment. Representative clonogenic assays showing similar colony numbers after treatment with LiCl are shown in the upper panel. Average of the absolute number of colonies in treatment groups are shown in the lower panel. Data is presented as the mean of three replicates of each experiment and the SEM compared using the Wilcoxon Rank-sum test.