Lithium and an EPAC-specific inhibitor ESI-09 synergistically suppress pancreatic cancer cell proliferation and survival

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Abstract

Our previous studies showed that while lithium suppresses proliferation and induces apoptosis in pancreatic cancer cells, the inhibition of exchange proteins directly activated by cyclic adenosine monophosphate (cAMP) (EPAC1) blocks pancreatic cancer cell migration and invasion. In this study, we further investigated the combinatory effects of lithium and EPAC-specific inhibitor (ESI)-09, an EPAC-specific inhibitor, on pancreatic cancer cell proliferation and viability, and explored whether lithium synergistically cooperates with EPAC inhibition in suppressing pancreatic cancer cell tumorigenicity. The cell viability of pancreatic cancer cell lines PANC-1 and MiaPaCa-2 was measured after 48 h of incubation with different dose combination of lithium and ESI-09. Flow cytometric analysis was carried out to further verify the impact of lithium and ESI-09 upon PANC-1 cell proliferation and apoptosis. To investigate the mechanism that the effects generated by lithium and ESI-09 on PANC-1 cells, the intracellular cAMP level was measured by an ELISA-based cAMP immunoassay. Our data showed that lithium and ESI-09 synergistically inhibit pancreatic cancer cell growth and survival. Furthermore, our results revealed a novel mechanism in which the synergism between lithium and ESI-09 is not mediated by the inhibitory effect of lithium toward GSK3β, but by lithium’s ability to suppress cAMP/protein kinase A signaling.

Key words: lithium, ESI-09, synergism, proliferation, apoptosis

Introduction

Pancreatic cancer has the lowest survival rate among all major cancers. Only 2%–10% of those diagnosed with pancreatic cancer will survive for more than 5 years. Pancreatic cancer is the seventh most common cause of cancer-related death in men and women across the world and the fourth leading cause of cancer-related death in the developed countries. There has been a 4-fold increase in the incidence of pancreatic cancer over the last 20 years. It is estimated that by 2020, there will be more than 418,000 newly diagnosed cases globally. According to the database from the National Central Cancer Registry of China (NCCR), the incidence of pancreatic cancer was 90,100 while 79,400 died of the disease in 2015 [1]. Characteristics of high malignancy, poor prognosis, and rapid metastasis make pancreatic cancer a disease with high mortality. Current treatment approaches for pancreatic cancer include surgical resection, radiation, and chemotherapy or combination therapy. However, these conventional therapeutic strategies have little impact on combating this aggressive neoplasm [2]. Novel therapeutic ideas
directly targeting the underlying pathophysiology and molecular biology are urgently needed.

Exchange proteins directly activated by cyclic adenosine monophosphate (cAMP) (EPAC) genes encode a family of cAMP-regulated guanine-nucleotide-exchange factors (GEFs). EPAC exhibits GEF activity toward Ras-like small GTPases, Rap1, and Rap2, after binding to the second messenger cAMP [3]. There are two isoforms of EPAC in human, named EPAC1 and EPAC2 [4]. EPAC1 and EPAC2 exhibit distinctive tissue expression patterns and play a crucial role in a myriad of physiological and pathological conditions. EPAC1 is ubiquitously expressed and rich in the heart and kidney. EPAC2 is mostly expressed in the central nervous system and endocrine glands [3,4]. The mRNA and/or protein levels of EPAC1 and EPAC2 are altered in certain types of diseases such as Alzheimer’s disease and pulmonary arterial hypertension [5,6]. Of particular importance, EPAC1 protein is significantly elevated in human pancreatic cancer cells as compared with normal pancreas or surrounding tissues [7]. Inhibition of EPAC1 by genetic knockdown or pharmacological inhibition suppresses pancreatic cancer cell migration and invasion in vitro and metastasis in vivo [8,9].

It has been almost 70 years since lithium was first described as a mood stabilizer [10]. After being approved by US Food and Drug Administration, lithium is widely used as an efficacious treatment of bipolar disorder and acute mania [11–13]. The therapeutic effects of lithium are typically attributed to its inhibitory activity toward GSK3β, which cross-talks with multiple key signaling transduce pathways [14,15]. In recent years, various studies have been carried out to explore the anti-tumor activity of lithium. In patients with bipolar disorder, lithium treatment is associated with a dose-dependent reduced overall cancer risk, particularly in patients with respiratory, gastrointestinal, and endocrine cancers [16,17]. Our recent study indicated that lithium inhibits pancreatic cancer cell proliferation, blocks G1/S cell-cycle progression, induces cell apoptosis and suppresses tumorigenic potential of pancreatic cancer cells through suppressing the hedgehog signaling pathway [18]. To explore the possibility that lithium synergistically cooperates with EPAC inhibition in suppressing pancreatic cancer cell tumorigenecity, here we tested the effect of combination treatment with lithium and ESI-09, an EPAC-specific inhibitor, on pancreatic cancer cell proliferation and viability.

Materials and Methods

Cell line and regents

The pancreatic ductal adenocarcinoma cell lines PANC-1 and MiaPaca-2 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum ( Gibco, Grand Island, USA) under 5% CO2 at 37°C. An additional 2.5% horse serum (Gibco) was added to the culture medium for MiaPaca-2 cells. CHO-K1-GLP1-R-CRE-Luc+ cells (GenScript, Piscataway, USA) were cultured in F12/DMEM supplied with 10% fetal bovine serum under 5% CO2 at 37°C. The CHO-K1-GLP1-R-CRE-Luc+ cell line is a modified CHO-K1 strain that stably expresses the glucagon-like peptide 1 receptor (GLP1-R) and also contains a luciferase reporter gene under the control of the cAMP response element (Hamster). Anti-GSK3β antibody, anti-phospho-GSK3β antibodies (Ser9, Tyr216), and anti-β-catenin antibody were purchased from Cell Signaling Technology (Beverly, USA). LiCl (CAS No. 7447-41-8) was obtained from EMD chemicals (Darmstadt, Germany), ESI-09 (CAS No. 263,707-16-0) and Exendin-4 (CAS No. 141,758-74-9) were purchased from Med Chem Express (Princeton, USA). CHIR99021 was purchased from Sigma (St Louis, USA). H89 (CAS No. 130,964-39-5) and gemcitabine (CAS No. 122,111-03-9) were purchased from Selleck (Houston, USA).

Cell viability assay

Cell viability was determined by CellTiter-Glo® 2.0 reagents (Promega, Madison, USA). Briefly, PANC-1 and MiaPaca-2 cells were suspended and seeded in the opaque-walled multiple-well plates (5000 cells/100 μl/well) and cultured overnight. Cells were treated with LiCl (0–10 mM), CHIR99021 (0–10 μM), ESI-09 (0–10 μM), gemcitabine (0–10 μM), and H89 (0–10 μM) alone, or treated with CHIR99021 (0–10 μM) + ESI-09 (0–10 μM), LiCl (0–10 mM) + ESI-09 (0–10 μM), and H89 (0–10 μM) + LiCl (0–10 mM) in combinations for 48 h. A total of 100 μl of CellTiter-Glo® 2.0 reagent was added to each well and incubated for 10 min. The luminescence intensity was recorded using a microplate reader (Thermo Electron Corp, Waltham, USA).

Analysis of synergetic effect

The synergetic effects between LiCl and ESI-09, CHIR99021 and ESI-09, or H89 and LiCl were evaluated by the combination index (CI) using the CompuSyn software program (Paramus, USA), which is based on the median effect principle [19]. The CI is calculated by the following formula: CI = D1/Dx1 + D2/Dx2, where (Dx1 and Dx2) indicate the individual dose of LiCl or CHIR99021 and ESI-09 required to inhibit a given level of cell growth, and D1 and D2 are the doses of LiCl or CHIR99021 and ESI-09 necessary to produce the same effect in combination, respectively [20]. CI = 1 is indicative for additive effect, whereas CI < 1 or CI > 1 suggests synergism or antagonism, respectively.

Cell apoptosis assay

Cell apoptosis was measured using apoptosis detection kit (Vazyme, Piscataway, USA), which was based on the labeling of the cells by Annexin-FITC and PI. The cells were treated with various concentrations of inhibitors for 24 h. Cells were then harvested and washed twice using phosphate-buffered saline (PBS), stained with Annexin-FITC and PI, and analyzed by flow cytometry (FACScan, BD, Franklin Lakes, USA) according to the manufacturer’s instructions. Data were analyzed using the FlowJo software program (Tree Star, Ashland, USA).

Cell proliferation assay

Cell proliferation was analyzed using CellTrace™ CFSE (5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester) reagent (Thermo Fisher Scientific, Shanghai, China). With the increasing generations of cell proliferation, the fluorescence signal decreased exponentially. Briefly, CFSE was added to the cell suspension in PBS and incubated for 20 min at room temperature in the dark. Then cells were seeded in a 96-well plate (5000 cells/well) and treated with indicated concentrations of compounds for 36 h. The cells were digested, collected, and analyzed by flow cytometry (FACScan) with 405 nm excitation and 450 nm emission. Data were analyzed by FlowJo software program. The cell division index was calculated using the following equation: Division index = (N(i+1)–N(i))/N(i) / 2; where i is the division number (undivided = 0) and N(i) is the number of events in division.
Intracellular cAMP determination
Intracellular cAMP levels were determined by an ELISA-based cAMP competitive immunoassay. All procedures were carried out according to the manufacturer’s instruction (GenScript).

Immunoblotting analysis
Total cellular protein extracts were obtained using radioimmunoprecipitation assay lysis buffer. Quantification of the cell lysates was determined using a bicinchoninic acid protein assay kit (Generay, Shanghai, China). After being blocked with 5% BSA in TBST (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween) at room temperature for 1 h, the PVDF membranes were incubated with the individual primary antibodies (1:5000) conjugated with horseradish peroxidase at room temperature for 1 h. The blots were detected using the enhanced chemiluminescence western blotting detection system (Millipore).

Statistics analysis
The results were presented as the mean ± SEM. Statistics significance of the experiments between the different groups was analyzed by one-way analysis of variance followed by the Student–Newman–Keuls comparisons. P < 0.05 was considered to be statistically significant.

Results
Effect of LiCl and ESI-09 on the cell viability of PANC-1 cells
The cell viability of PANC-1 was assessed after 48 h of incubation with different concentrations of LiCl and ESI-09. As shown in Fig. 1A, LiCl inhibited the viability of PANC-1 cells in a dose-dependent manner. Similarly, ESI-09 also inhibited PANC-1 cell viability dose-dependently. Combination of LiCl and ESI-09 showed a significantly larger inhibitory effect toward PANC-1 cells, compared with LiCl or ESI-09 alone. The CI value quantitatively reflects the pharmacological interaction of two drugs. As shown in Fig. 1B, the CI values of all the test concentrations were well-below 1, which indicated that ESI-09 synergized with LiCl to inhibit PANC-1 cell viability. Similar results were obtained when pancreatic cell line MiaPaca-2 was treated with the combination of LiCl and ESI-09, suggesting that ESI-09 synergized with LiCl to inhibit Miapaca-2 cell viability (Fig. 1C,D). As expected, cell viabilities of both PANC-1 cells and MiaPaCa-1 cells were dose-dependently inhibited by gemcitabine, a FDA approved front-line treatment for pancreatic cancer, and the inhibitory efficacy of ESI-09 and LiCl combination was comparable to gemcitabine alone.

Figure 1. LiCl, ESI-09, and combination of LiCl and ESI-09 treatment inhibited PANC-1 cell viability (A) PANC-1 cells were treated with different concentrations of LiCl (0–10 mM), ESI-09 (0–10 μM) alone, or with combination of LiCl and ESI-09 (0, 1 mM + 1 μM, 3 mM + 3 μM, 5 mM + 5 μM, and 10 mM + 10 μM) for 48 h. (B) Combination index (CI) analysis of growth inhibition in PANC-1 cells after 48 h of incubation with LiCl and ESI-09. Data from (A) were converted to fraction affected (Fa) and plotted against CI. Dotted line on the graph designated a CI equal to 1. (C) MiaPaCa-2 cells were treated with different concentrations of LiCl (0–10 mM), ESI-09 (0–10 μM) alone, or with combination of LiCl and ESI-09 (0, 1 mM + 1 μM, 3 mM + 3 μM, 5 mM + 5 μM, and 10 mM + 10 μM) for 48 h. (D) CI analysis of growth inhibition in MiaPaCa-2 cells after 48 h of incubation with LiCl and ESI-09. Data from (C) were converted to Fa and plotted against CI. Dotted line on the graph designated a CI equal to 1. The combination of the two agents was compared with LiCl alone. ****P < 0.001, ***P < 0.005, **P < 0.01, and *P < 0.05. The combination of the two agents was compared to ESI-09 alone. ####P < 0.001, ####P < 0.005, ####P < 0.01, and ####P < 0.05. Similar results were obtained from three independent experiments.
superior to gemcitabine treatment for both cell lines (Supplementary Fig. S1).

ESI-09 and LiCl synergistically inhibited the cell proliferation of PANC-1 cells

Our previous studies showed that LiCl treatment suppressed pancreatic cancer cell proliferation and blocked G1/S cell-cycle progression [20]. Consistent with these observations, PANC-1 cells treated with different doses of LiCl resulted in a dose-dependent decrease of division index (Fig. 2). The division index for 10 mM LiCl treatment is around 0.56. Similarly, ESI-09 treatment also resulted in a dose-dependent reduction of division index, showing a division index of 0.38 at 10 μM. The division index of the combination treatment of 10 mM LiCl and 10 μM LiCl was 0.003, suggesting a strong synergism (CI < 0.6) between LiCl and ESI-09 in inhibiting PANC-1 cell proliferation.

Effect of LiCl and ESI-09 treatment on PANC-1 cell apoptosis

Apoptosis induced by LiCl and ESI-09 was further evaluated by Annexin V and propidium iodide staining. Treatment of PANC-1 cells with either drug for 24 h resulted in cell apoptosis in a dose-dependent manner (Fig. 3A). Increased percentage of early apoptotic cells (Fig. 3B) and late apoptotic cells (Fig. 3C) was observed after combined treatment with LiCl and ESI-09. These data suggested that the combination of LiCl and ESI-09 synergistically (CI < 1) induced PANC-1 cell apoptosis.

Synergism between LiCl and ESI-09 is not dependent on GSK3β inhibition

To investigate if the apparent synergism observed between LiCl and ESI-09 is mediated by LiCl’s effect on GSK3β inhibition, a GSK3β-specific inhibitor, CHIR99021 was tested in parallel. As shown in Fig. 4A, both CHIR99021 and Li inhibited GSK3β, as reflected by the reduction in phospho-GSK3β (Ser9 and Tyr216) as well as the increase of the protein level of β-catenin, since the inhibition of GSK3β is known to suppress the ubiquitin-mediated degradation of β-catenin.

While CHIR99021 alone also inhibited the viability of PANC-1 cells (Fig. 4B), no synergism was observed between CHIR99021 and ESI-09, as the CI values were close to or above 1 at all concentrations tested (Fig. 4C,D). Taken together, these data suggested that the apparent synergism observed between LiCl and ESI-09 is not dependent on LiCl’s ability to inhibit GSK3β.

LiCl, but not CHIR99021, inhibited intracellular cAMP production

In addition to inhibiting GSK3β, lithium is also known to inhibit cAMP production and protein kinase A (PKA) activation [21]. Consistent with these notions, treatment of PANC-1 cells with LiCl reduced the basal intracellular cAMP concentration dose-dependently (Fig. 5A), while CHIR99021 had no such effect (Fig. 5B). Furthermore, cells treated with 10 mM LiCl significantly inhibited Exendin-4-induced luciferase expression under the control of CRE-dependent promoter in CHO-K1-GLP-1 R-CRE-Luc+ cells (Fig. 5C), whereas 10 μM CHIR99021 had no effects (Fig. 5D).
Figure 4. CHIR99021, ESI-09, and combination of CHIR99021 and ESI-09 inhibited the viability of PANC-1 cells
(A) Total cellular GSK3β and phosphorylated GSK3β (Ser9, Tyr216) after treatment with 10 mM LiCl or 10 μM CHIR99021 for 24 h, and β-catenin accumulated since the two compounds inhibited the ubiquitylated degradation by suppressing GSK3β. (B) PANC-1 cells treated with different concentrations of CHIR99021 (0–10 μM) for 48 h. (C) PANC-1 cells treated with different concentrations of combination of CHIR99021 and ESI-09 (0, 1 μM + 1 μM, 3 μM + 3 μM, 5 μM + 5 μM, and 10 μM + 10 μM) for 48 h. (D) Combination Index (CI) analysis of growth inhibition in PANC-1 cells after 48 h of incubation with CHIR99021 and ESI-09. Data from (C) were converted to Fa and plotted against CI. Dotted line on the graph designated a CI equal to 1. Similar results were obtained from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.0001.

Figure 3. Combination of LiCl and ESI-09 induced apoptosis of PANC-1 cells
(A) A significant increase in apoptosis was detected by AV/PI double staining in PANC-1 cells at 24 h after LiCl and ESI-09 treatment alone or in combination. (B) Early apoptotic cell percentage of PANC-1 cells at 24 h after LiCl and ESI-09 treatment alone or in combination. (C) Late apoptotic cell percentage of PANC-1 cells at 24 h after LiCl and ESI-09 treatment alone or in combination. The combination of the two agents was compared with LiCl alone. ***P < 0.005, **P < 0.01, and *P < 0.05. The combination of the two agents was compared to ESI-09 alone; ****P < 0.005, ***P < 0.01, and **P < 0.05. Similar results were obtained from three independent experiments.
These results suggested that LiCl, not CHIR99021, inhibited intracellular cAMP/PKA signaling.

**Discussion**

Cyclic AMP is a universal stress signal that plays a fundamental role in a multitude of physiological and pathophysiological responses. In eukaryotic cells, the effects of cAMP are mainly mediated by two ubiquitously expressed receptors, the well-known PKA and the EPAC. Under various physiological and pathological conditions, PKA and EPAC may exert opposite or synergistic effects on cellular functions in a spatial and temporal manner [22–24]. Dysregulation of the cAMP signaling pathway is common in various cancers and affects disease progression and treatment options [25–29]. While genetic mutations of PKA have been shown to directly contribute to the development of cancers in patients with Carney complex, fibrolamellar hepatocellular carcinoma, and Cushing’s disease [30–34], the role of EPAC in cancers is less clear [35].

The involvement of cAMP signaling pathway in pancreatic cancer, one of the most aggressive and deadly human neoplasia, has been reported [36]. For example, inhibition of PKA in pancreatic cancer cells induces growth arrest and apoptosis [37]. Furthermore, it has been shown that the expression of EPAC1 is upregulated in pancreatic cancer [7], and inhibition of EPAC suppresses pancreatic cancer cell migration and invasion [8,9,38]. These studies suggested that manipulations of the cAMP signaling pathway may represent viable strategies for the prevention and adjuvant therapy targeting pancreatic cancer. In fact, it has been demonstrated that suppressing cAMP production by the beta-blocker propranolol has strong preventive effects on the development of pancreatic cancer in animal models [39].

Our previous studies showed that lithium inhibits tumorigenic potential of pancreatic ductal adenocarcinoma (PDAC) cells through targeting Hedgehog-GLI signaling, a known down-stream effector pathway of PKA. In this study, we sought to target pancreatic cancer by combining lithium and EPAC inhibition treatment. Using a novel ESI, ESI-09, which is identified through a high-throughput screening [40] and is capable of selectively inhibiting the functions of EPAC in vitro and in vivo [8,41,42], our results showed that either LiCl or ESI-09 alone could induce pancreatic cancer cell growth arrest and apoptosis. Importantly, the combination of the two agents synergistically inhibited pancreatic cancer cell proliferation and survival. It is well-known that many of the cellular and therapeutic effects of lithium are mediated by its ability to inhibit...
GSK3β, an important molecular switch involved in many key signaling pathways [43–45]. Surprisingly, the synergy between lithium and ESI-09 is not due to GSK3β inhibition, as substitution of lithium with CHIR99021, a GSK3β-specific kinase inhibitor, did not show any synergistic effect when used in combination with ESI-09 [46]. In addition to inhibiting GSK3β, lithium can also block cAMP/PKA signaling by suppressing the adenylate cyclase activity [47,48]. Indeed, treatment of pancreatic cancer cells with lithium could lead to significant decreases of intracellular cAMP and PKA pathway activation. These results, coupled with the finding that combination treatment of H89 and ESI-09 also showed a synergistic inhibitory effect toward PANC-1 cells, revealed a novel mechanism in which lithium, through its ability to suppress cAMP/PKA signaling, synergistically cooperates with ESI-09 in inhibiting pancreatic cancer cell proliferation and survival.

**Supplementary Data**

Supplementary data is available at Acta Biochimica et Biophysica Sinica online.

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**References**


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**Figure 6. H89 and combination of H89 and ESI-09 inhibited the viability of PANC-1 cells**  
(A) PANC-1 cells treated with different concentrations of H89 (0–10 μM) for 48 h. (B) PANC-1 cells treated with different concentrations of combination of H89 and ESI-09 (0, 1 μM+1 μM, 3 μM+3 μM, 5 μM+5 μM, and 10 μM+10 μM) for 48 h. (C) Combination index (CI) analysis of growth inhibition in PANC-1 cells after 48 h of incubation with H89 and ESI-09. Data from (B) were converted to Fa and plotted against CI. Dotted line on the graph designated a CI equal to 1. Similar results were obtained from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.0001.