Inhibition of cell proliferation by lithium is associated with interference in cdc2 activation

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Abstract Lithium can interfere with embryonal development in a variety of organisms. We investigated the effect of lithium on the proliferation of early embryonal cells. [^{3}H]Thymidine incorporation of non-committed mouse P19 embryonal carcinoma cells was inhibited by lithium treatment. Similar effects were seen in a variety of other cells. This growth inhibition occurred in the G_2 phase, since cells accumulated with a 4N DNA content, but the appearance of mitotic cells was blocked. Lithium could also prevent the activation of cdc2, thereby inhibiting cyclin B/cdc2 kinase activity. These data indicate that lithium might disturb embryonal development through interference in embryonal cell cycle regulation.

Key words: Cell cycle; Checkpoint; Cyclin; G_2; Lithium

1. Introduction

Early embryonal stem cells undergo rapid cycles of cell division, but the cell cycle lengthens as cells become more committed. This lengthening is due to the introduction of cell cycle control mechanisms, causing the cells to pause between the alternating S and M phases, in G_1 or G_2 [1]. During G_2, initiation of mitosis is prevented by inhibitory phosphorylation of the mitotic cyclin B/cdc2 complexes. This inhibitory phosphorylation takes place on the Thr-14 and Tyr-15 residues of cdc2 and is mediated by Wee1 and related kinases [2–5]. At the end of the G_2 phase, the phosphatase Cdc25C dephosphorylates these inhibitory sites, triggering the activation of cdc2 [6,7].

Lithium is used as a treatment for bipolar psychiatric disorders [8]. In addition, it has been demonstrated that lithium can stimulate proliferation of some cells [9] and has teratogenic effects [10,11], when used at concentrations applied in human therapy. For example, in *Xenopus* embryos, lithium works as a modifier of mesoderm induction that can induce duplication of the dorsal axis [10]. In *Dictyostelium*, lithium influences cell fate determination by blocking spore cell and promoting stalk cell development [11].

In this paper, we demonstrate that lithium inhibits the proliferation of mouse embryonal cells, as well as in a variety of other cell lines. The effect of lithium is exerted in the G_2 phase and is associated with an inhibition of cyclin B-associated kinase activity, due to retention of phosphorylation on the Tyr-15 regulatory site of cdc2.

2. Materials and methods

2.1. Cell culture, antibodies and reagents

U_2OS (ATCC), A14 [12] and Rat-1 (ATCC) cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco). P19 cells (ATCC) were also cultured in DMEM, on gelatin-coated dishes. SK-N-MC (ATCC) cells were cultured in a 1:1 mixture of DMEM and Ham's F12 (DF12 medium, Gibco). The culture media were supplemented with 10% fetal calf serum (FCS, Integro), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For differentiation, P19 cells were grown in aggregates on bacteriological petri dishes in the presence of retinoic acid (RA) 10^{-6} M for 5 days ($P19^{AG}$+RA) [13]. Cells were arrested at the metaphase/anaphase transition of the M phase by treating the cells with nocodazole (2.5 μg/ml; Sigma) for 16 h [14]. To arrest the cells at the G_1/S transition, cells were treated with thymidine (2.5 mM; Sigma) for 24 h. Cells were released from a thymidine block by washing twice with pre-warmed PBS, followed by washing twice with pre-warmed culture medium for 15 min at 37°C.

Protein A/G plus agarose, and the mouse monoclonal antibodies against cyclin B1 (GNS1) and cdc2 (clone 17) were purchased from Santa Cruz. Polyclonal phosphospecific cdc2-Tyr-15 antibody was obtained from New England Biolabs. Histone H1 was obtained from Boehringer Mannheim. Propidium iodide was purchased from Sigma.

2.2. [^{3}H]Thymidine incorporation assay and cell cycle analysis

[^{3}H]Thymidine incorporation was performed as described [15].

For DNA profiles, the cells were grown with or without LiCl (10 mM) for 48 h, after which cells were harvested and fixed overnight in 70% ethanol at 4°C. After washing away the ethanol, the cells were stained with propidium iodide in a solution containing 10 μg/ml propidium iodide and 10 μg/ml DNase-free RNase. The stained cells were analyzed on a fluorescence activated cell sorter (FACS) using Lysis II software flow cytometry analysis (Becton Dickinson).

To determine the percentage of mitotic cells, cells were harvested, fixed in 70% ethanol and cytospins were prepared. DNA was stained using 4′,6-diamidino-2-phenylindole (DAPI, 20 μg/ml) [16]. The percentage of mitotic figures was scored using fluorescence microscopy (≥200 cells per time point).

2.3. Western blotting, immunoprecipitation and in vitro kinase reactions

For Western blotting, cells were lysed directly in Laemmli sample buffer without β-mercaptoethanol or bromophenol blue and the samples were boiled for 5 min. Protein concentrations were determined using the Lowry protein assay [17]. Subsequently, β-mercaptoethanol and bromophenol blue were added. Proteins were separated on a polyacrylamide (PAA) gel and blotted to a nitrocellulose membrane. Proteins were detected with antibody incubation (mouse monoclonal anti-cdc2, recognizing human, mouse and *Xenopus* cdc2, non-cross-reactive with other cdks; rabbit polyclonal phosphospecific anti-cdc2-Tyr-15) overnight at 4°C. This was followed by the secondary antibody for 1 h at room temperature. The blots were developed using enhanced chemiluminescence (ECL).

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; FACS, fluorescence activated cell sorter; GSK-3, glycogen synthase kinase 3; IMPase, inositol monophosphatase; PAA, polyacrylamide; RA, retinoic acid

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For immunoprecipitation, the cells were washed with ice-cold PBS and lysed in NETN (400 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 0.5% NP-40 supplemented with 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_2VO_3, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml trypsin inhibitor) for 30 min at 4°C. Lysates were centrifuged at 15 000 rpm at 4°C. Cyclin B1 was immunoprecipitated with 0.3 μg of anti-cyclin B1 antibody and protein A/G plus agarose for 4 h to overnight at 4°C. Subsequently, immmunoprecipitates were washed three times with lysis buffer. For cdc2 immunoblotting, precipitates were washed once again with lysis buffer, after which sample buffer was added. Then the samples were boiled for 5 min, the proteins were separated on a 12% PAA gel and cdc2 was detected by Western blotting. In vitro kinase reactions were performed as described [15].

3. Results

3.1. Lithium inhibits proliferation of P19 cells

To study if lithium can affect the proliferation of early embryonal cells, we used mouse P19 embryonal carcinoma cells as a model for embryonal cells [18]. We found that proliferation was dramatically inhibited, as determined by [^{3}H]thymidine incorporation (Fig. 1A). To determine whether this effect was restricted to non-committed early embryonal cells, we analyzed the effect of lithium on proliferation in differentiated P19 cells. To this end, P19 cells were cultured in aggregates in the presence of RA for 5 days, inducing mesodermal and neuronal differentiation [13]. Treating such differentiated P19 cells for 24 h or 48 h with LiCl resulted in a decrease of [^{3}H]thymidine incorporation of ∼30% and ∼45%, respectively (Fig. 1A). Although the effect of lithium is not as dramatic in the differentiated P19 cells compared to the undifferentiated cells, we do not think this is due to a difference in sensitivity to lithium but rather to the slower proliferation rate of the differentiated cells.

In addition, we examined the dose dependence of growth inhibition by lithium. As shown in Fig. 1B, proliferation is already inhibited at a concentration of 0.5 mM LiCl.

To determine in which phase of the cell cycle lithium exerts its growth-inhibitory effect, we analyzed cell cycle profiles of asynchronously growing P19 cells, which were treated with LiCl for 48 h. Treatment with LiCl resulted in an increase in the number of cells with a 4N DNA content from 26% in untreated cells to 47% in LiCl-treated cells (Fig. 1C). Also differentiated P19 cells showed an accumulation of cells in the G_2/M phases upon LiCl treatment (data not shown).

3.2. Lithium induces growth inhibition in several cell lines

To analyze if the effect of lithium on G_2/M progression is more general, we investigated its effect on the proliferation of a diverse range of cell lines. The effect of lithium on DNA synthesis in U_2OS cells (human osteosarcoma), SK-N-MC cells (human neuroepithelioma), Rat-1 cells (rat fibroblast) and NIH 3T3-derived A14 cells (mouse fibroblast) was determined by [^{3}H]thymidine incorporation in the absence or presence of LiCl. Treatment of U_2OS, SK-N-MC and A14 cells with LiCl resulted in a decrease of [^{3}H]thymidine incorporation from ∼45% in SK-N-MC cells to ∼60% in U_2OS cells and up to ∼75% in A14 cells (Fig. 2A). [^{3}H]Thymidine incorporation in Rat-1 cells was not influenced by LiCl treatment (Fig. 2A).

Also in these cells the growth inhibition occurred in G_2/M. Treating U_2OS cells with LiCl for 24 h resulted in an increase in the 4N population from 20% to 32% (data not shown), whereas treatment for 48 h resulted in an increase up to 49% (Fig. 2B). Treatment of SK-N-MC cells and A14 cells with LiCl also resulted in an increase in the amount of cells in the G_2/M phase (data not shown), indicating that lithium has a similar effect on a diverse range of cell lines.

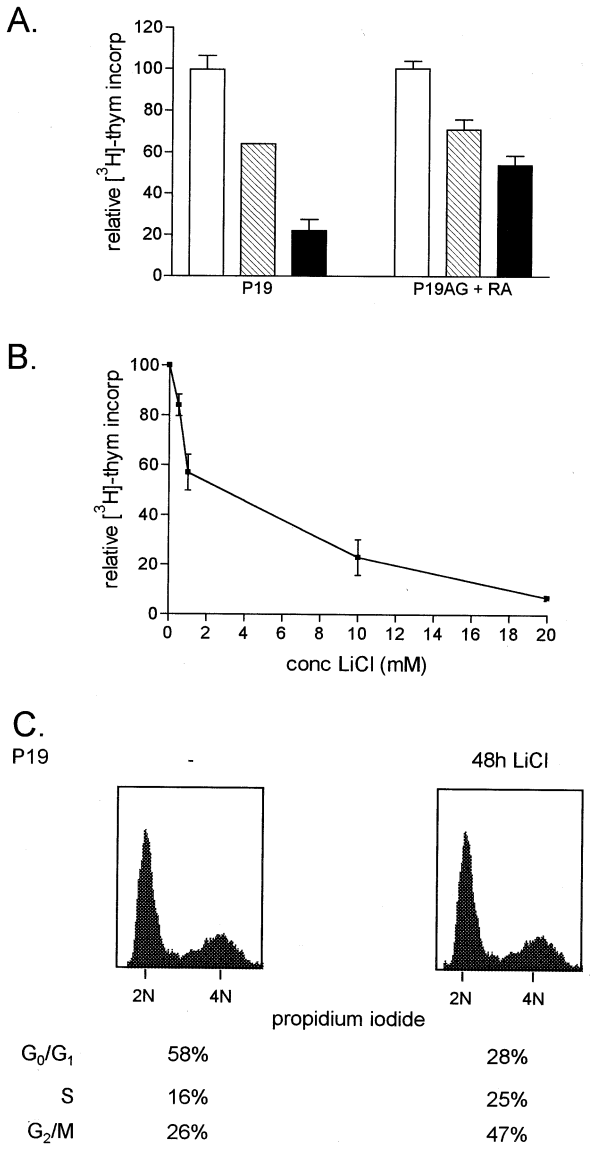

Fig. 1. Lithium inhibits proliferation of P19 cells by a block in G_2/M. A: Asynchronously growing P19 cells or differentiated P19 cells ($P19^{AG}$+RA) were seeded in 24-well dishes at 40 000 cells per well. After 24 h, the cells were left untreated (white bars) or treated with LiCl (10 mM) for 24 (gray bars) or 48 h (black bars). [^{3}H]Thymidine incorporation was determined in triplicate and is represented as percentage incorporation of untreated cells (actual, P19: 14×10^5 cpm/well; $P19^{AG}$+RA: 0.7×10^5 cpm/well). Error bars represent the S.E.M. B: Asynchronously growing P19 cells were seeded in 24-well dishes at 10 000 cells per well. After 24 h, the cells were treated with different concentrations of LiCl for 24 h. [^{3}H]Thymidine incorporation was determined as described in A. C: Asynchronously growing P19 cells were left untreated or treated with LiCl (10 mM). After 48 h, the cells were harvested and cell cycle profiles were obtained by staining with propidium iodide.

3.3. Lithium inhibits G_2 phase progression, not mitosis

To determine whether lithium-treated cells are blocked in

the G_2 phase or in M phase, we tested whether lithium could inhibit cells from entering mitosis. To this end, U_2OS cells were treated with nocodazole, to arrest them at the metaphase/anaphase transition of the M phase, in the presence or absence of LiCl. At several time points, cells were harvested and the percentage of mitotic cells was determined by immunofluorescence staining with the DNA-interchelating dye DAPI. In the samples treated with nocodazole, the first mitotic cells appeared within 6 h (Fig. 3). The percentage of mitotic cells increased with time and after 16 h a large percentage of the cells (~56%) was in the M phase. In contrast, cells treated with nocodazole and LiCl clearly showed a delay in entering mitosis. After 16 h, we could only detect ~12% mitotic cells.

In addition, U_2OS cells were synchronized at the G_1/S transition using thymidine. When the cells were released in the presence of LiCl the appearance of mitotic cells was delayed compared to the untreated cells (data not shown), again indicating that the block occurs prior to initiation of mitosis.

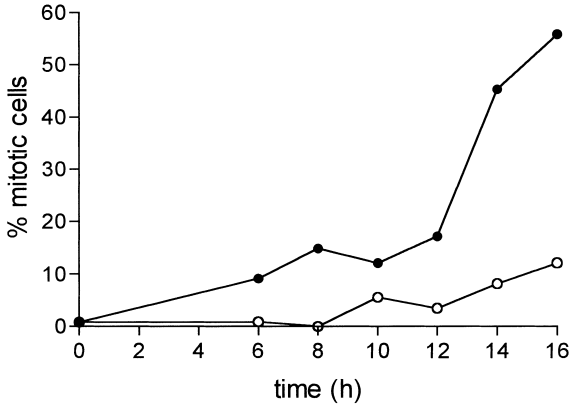

Fig. 3. Lithium inhibits M phase entry. U_2OS cells were treated with nocodazole in the absence (closed circles) or presence (open circles) of LiCl (10 mM). At different time periods, cells were harvested. Then, DNA was stained using DAPI. The percentage of mitotic cells was determined by scoring for cells with condensed chromosomes, using immunofluorescence microscopy.

3.4. Lithium prevents the activation of cdc2

For entering M phase, kinase activity of the mitotic cyclin B/cdc2 complex is required [3]. We therefore investigated if lithium could interfere with the activation of the cyclin B/cdc2 kinase. To do so, U_2OS cells were synchronized at the G_1/S transition using thymidine. Next, the cells were released in the presence or absence of LiCl, and nocodazole was added, to ensure that the synchronized cells eventually block at the metaphase-anaphase transition of the M phase. At different time points after the release, we measured cyclin B-associated kinase activity (Fig. 4A). Kinase activity in the untreated samples rose 16 h after the release and increased with time. In contrast, we observed only a minor increase in cyclin B-associated activity in cells released from the thymidine block in the presence of LiCl. This increase occurred at a later time point and the overall kinase activity remained low.

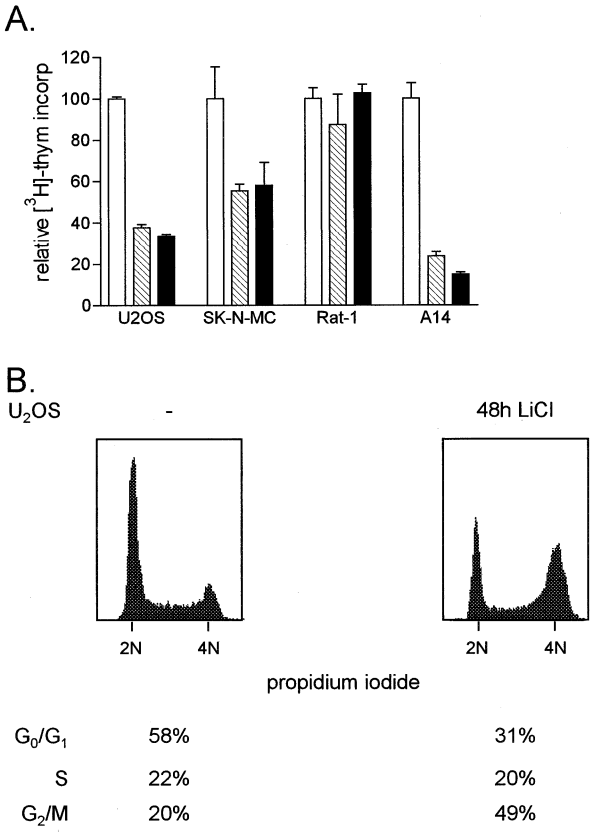

Fig. 2. Lithium inhibits proliferation of A14, U_2OS and SK-N-MC cells, but not Rat-1 cells. A: U_2OS, SK-N-MC, Rat-1 and A14 cells were seeded in 24-well dishes at 40 000 cells per well. After 24 h, the cells were left untreated (white bars) or treated with LiCl (10 mM) for 24 (gray bars) or 48 h (black bars). [^{3}H]Thymidine incorporation was determined in triplicate and is represented as percentage incorporation of untreated cells (actual, 1.2×10^5 to 4.2×10^5 cpm/well). Error bars represent the S.E.M. B: U_2OS cells were left untreated or treated with LiCl (10 mM) for 48 h. Then, the cells were harvested and cells cycle profiles were obtained by staining with propidium iodide.

The reduction in cyclin B/cdc2 kinase activity was not due to inhibition of cyclin B expression, since cyclin B protein levels were not influenced by lithium treatment (data not shown).

We next analyzed the phosphorylation state of cdc2 after treatment with lithium. Cyclin B immunoprecipitations obtained at the same time points after release from the thymidine block were subjected to immunoblotting for cdc2. Dephosphorylation of the Tyr-15 residue of cdc2 leads to the activation of cdc2 at the G_2/M transition and results in a mobility shift on PAA gels [19]. In cells blocked at the G_1/S transition using thymidine, only the slower migrating form of cdc2 could be detected (Fig. 4B). This form represents the inactive form of cdc2, phosphorylated on Tyr-15 [19], which we confirmed with a phosphospecific cdc2-Tyr-15 antibody (Fig. 4B). Activated cdc2 was present after a 13 h release in the absence of LiCl and the amount of Tyr-15-dephosphorylated protein increased with time. Releasing the cells in the presence of LiCl showed that cyclin B/cdc2 complex formation occurred normally, but the Tyr-15 dephosphorylation was inhibited. After 25 h ~80% of the cdc2 is in its activated, faster migrating form in untreated samples, whereas in the samples treated with LiCl, only ~20% of the cdc2 is active.

As is shown in Fig. 4C, the inhibition of Tyr-15 dephosphorylation of cdc2 shows a very similar concentration dependence for LiCl as was observed for the inhibition of [^{3}H]thymidine incorporation (Fig. 1B).

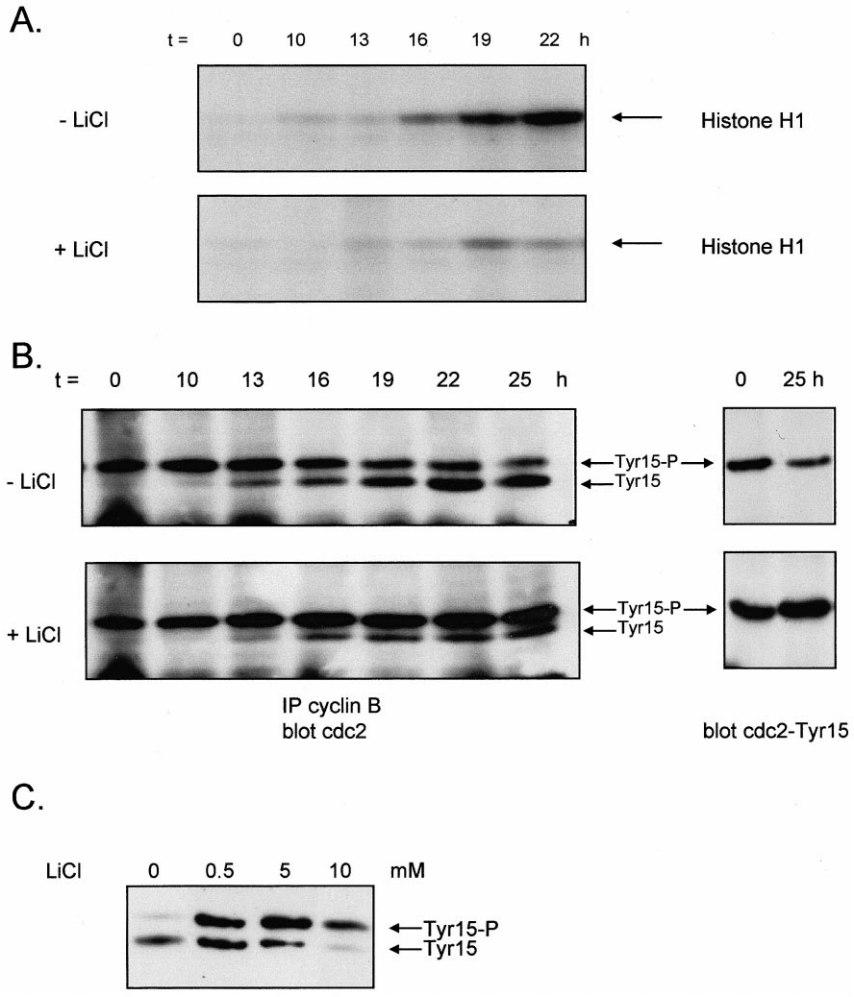

Fig. 4. Lithium inhibits cyclin B/cdc2 activation. A: U_2OS cells were blocked at the G_1/S transition by thymidine treatment for 24 h. Thymidine was washed away and the cells were released in the presence of nocodazole and in the absence or presence of LiCl (10 mM). At different time periods, the cells were lysed. Cyclin B was immunoprecipitated and cyclin B-associated kinase activity was determined using an in vitro kinase reaction with histone H1 as substrate. 5 μg of protein was used in the immunoprecipitation, plateau of the kinase assay at 50 μg. Assays were performed in duplicate and the variation never exceeded 5% (as determined by phosphor imager). B: As A, but after cyclin B immunoprecipitation, cdc2 was detected by immunoblotting. The faster migrating form is the active, Tyr-15-dephosphorylated form of cdc2 (left panel). In total lysates, cdc2-Tyr-15-P was detected by Western blotting with a phosphospecific anti-cdc2-Tyr-15 (right panel). C: Asynchronously growing U_2OS cells were treated with nocodazole in the presence of different concentrations of LiCl. After 16 h, the cells were lysed, cyclin B was precipitated, and cdc2 was detected by immunoblotting. The faster migrating form is the active, Tyr-15-dephosphorylated form of cdc2.

4. Discussion

In this paper, we show that lithium induces a G_2 arrest in a variety of cell lines and inhibits the appearance of cyclin B/cdc2 kinase activity. This inhibition is due to retention of inhibitory phosphorylation of Tyr-15 on cdc2. Dephosphorylation of cdc2-Tyr-15 is a critical step in the activation of the cyclin B/cdc2 complex [6,7]. Our results suggest that lithium might prevent the activation of the mitotic cyclin B/cdc2 complex, through interference with Tyr-15 dephosphorylation.

Two different pathways have been described by which lithium could affect embryonal development. Lithium has been shown to inhibit inositol monophosphatase (IMPase) in vitro [20,21] and lithium can act as an inhibitor of glycogen synthase kinase 3 (GSK-3) and thereby mimic Wingless signaling [22,23]. Whether one of these pathways is involved in the effect of lithium we describe here is currently unknown.

DNA damage will also result in an arrest in G_2 progression and this arrest is caused by inhibition of cdc2 dephosphorylation [24]. Recent studies showed that the inhibition of cdc2 activation after DNA damage is mediated by the inactivation of Cdc25C [25,26] through phosphorylation-dependent binding to 14-3-3 [27–29]. Present cancer therapies mostly rely on the increased sensitivity of tumor cells to a variety of DNA-damaging agents. Since lithium seems to interfere with the same pathway as these agents, it will be of significant interest to identify the target(s) of lithium and to investigate the relative sensitivity of normal and tumor cells to lithium treatment. In that respect, it is very interesting that lithium has been reported to reduce the incidence of cancer [30].

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