Abstract. Lithium is the main therapeutic agent for the treatment of bipolar disorders but nerve cells are not the sole target of this drug. Indeed, lithium has been reported to target numerous cell types and to affect cell proliferation, differentiation and death. Lithium targets a variety of enzymes among which there is GSK-3ß and a number of cell responses elicited by lithium are mediated by the Wnt pathway that is involved in medulloblastoma (MB) pathogenesis. We studied the in vitro effects of lithium on two different MB cell lines: D283MED and DAOY. High doses of lithium inhibited GSK3-ß, decreased cell proliferation and induced non-apoptotic cell death in both cell lines independently by intracellular levels of ß-catenin that is consistently high only in D283MED. At clinical doses, the anti-neoplastic effects were observed only in this cell line, highlighting the importance of a specific molecular background in determining the target therapy response. In conclusion, lithium could be a promising drug in MB, but an accurate molecular profile predictive of drug response still needs to be clarified.

Introduction

Lithium is known to target nerve cells and is still being used for the treatment of nervous disorders (1). However, a multiplicity of cell responses is elicited by lithium outside the nervous system and has profound effects on a variety of processes, including metabolism, cell proliferation and development (2,3). In particular, lithium is described as an inhibitor of the cell cycle (4-7) and an accelerator of cell death (8-10). Although some of these data may be debatable, the idea is thus raised that lithium may have antitumor properties. These effects are however cell-type dependent: on the one hand, lithium stimulates cell proliferation in mammary tumour cells (11), and on the other, it inhibits cell proliferation in melanoma (12), hepatocellular carcinoma (13) and prostate cancer (7), and it blocks glioma cell migration and invasion in vitro (14). Lithium chloride can also induce apoptosis in several cell types (8,15), even if it has been reported to have anti-apoptotic, especially neuroprotective, effects in certain experimental settings (16-19). Lithium acts by targeting various signalling pathways and cellular enzymes: a central action of lithium is a non-ATP competitive inhibition of glycogen synthase kinase-3ß (GSK-3ß) (20). GSK-3ß belongs to a family of highly conserved serine/threonine kinase and is involved in many biological processes, including cell cycle progression, gene transcription, apoptosis/survival, cellular metabolism, cell movement, tumorigenesis, cytokinesis and embryonic development (20-23). Lithium mimics the effects of Wnt signalling activation by inhibiting the activity of GSK-3ß, which is also involved in the degradation of ß-catenin. Activation of the canonical Wnt pathway stabilizes cytosolic ß-catenin, which translocates to the nucleus and then stimulates T-cell and lymphoid enhancer factors, leading to upregulation of different target genes (24). Several studies have reported that nuclear ß-catenin is an independent marker of a good outcome in medulloblastoma (MB), an embryonal neuroepithelial tumour of the cerebellum and the most common malignant brain tumour of childhood (25-27). Together, these data suggest that lithium, which is a potent regulator of the Wnt pathway, may interfere with the mechanisms of cell proliferation in MB. Thus, in this study, the effect of lithium was evaluated on two MB cell lines: D283MED, with a p53 wild-type, and DAOY, harbouring a p53 mutation (28).

Materials and methods

Cell lines, culture conditions and lithium treatment. Human MB cell lines (D283MED and DAOY) were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cell lines were maintained as exponentially growing cultures in Eagle’s minimum essential medium (EMEM) with...
Earle's BSS (Invitrogen Corporation; Frederick, MD) supplemented with 20% heat-inactivated foetal bovine serum (FBS, Invitrogen Corporation), 1% L-glutamine (Eurobio; Courtaboeuf, France), 0.5% gentamycin (Euroclone Life Sciences Division; Milan, Italy), 0.5% neomycin (Sigma-Aldrich; St. Louis, MO), 1% non-essential amino acids (Eurobio) and 1% sodium pyruvate (Eurobio). Cell cultures were maintained at 37°C in 5% CO₂. The cells were plated 24 h before experiments and then LiCl (Sigma-Aldrich) was added to the culture medium at a dose of 20 mM. Cells were analyzed to study early and late responses to the treatment by evaluating: inhibition of GSK-3β, nuclear β-catenin expression and β-catenin localization, cell proliferation, cell cycle, cell mortality and apoptosis. Moreover, cell mortality was assessed for doses of lithium <20 mM: 0.5, 1 and 10 mM.

**Protein expression analysis.** D283MED and DAOY cells were harvested 8, 16, 24, 29, 40 and 48 h after the addition of LiCl and total protein extracts were prepared as previously described (29), using the following extraction buffer: 1% Triton, 10% glycerol, 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA pH 8.0, 2 mM MgCl₂, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and Complete EDTA-free (Roche Diagnostic; Indianapolis, IN) 2X.

Nuclear proteins were extracted 24, 29, 32, 40 and 48 h after the addition of LiCl, as previously described (29). Aliquots of nuclear pellets were stained with hematoxylineosin (HE) and observed under a light microscope (Carl Zeiss GmbH; Oberkochen, Germany) to verify the lack of cytoplasmic contamination. Western Blotting (WB) was performed as previously described (29), using the following primary antibodies at the respective dilutions in blocking solution: rabbit monoclonal anti-GSK-3β (27C10, Cell Signaling Technology; Danvers, MA) 1:1000; rabbit polyclonal anti-phospho-GSK-3β (Ser9, Cell Signaling Technology) 1:1000; goat polyclonal anti-β-actin (I-19, Santa Cruz Biotechnology; Santa Cruz, CA) 1:1000; rabbit polyclonal anti-β-catenin (H-102, Santa Cruz Biotechnology; Santa Cruz, CA) 1:1000; rabbit polyclonal anti-β-catenin (H-102, Santa Cruz Biotechnology) 1:600; goat polyclonal anti-lamin B (C-20, Santa Cruz Biotechnology) 1:1000; mouse monoclonal anti-HSP90 (SPA-830, Stressgen Bioreagents; Ann Arbor, MI) 1:100. Primary antibodies were detected with horseradish peroxidase (HRP)-labelled secondary antibodies at the respective dilutions in blocking solution: anti-rabbit (NA9343, GE Healthcare Europe GmbH; Freiburg, Germany) 1:10000; anti-goat (sc-2020, Santa Cruz Biotechnology) 1:1000. Total GSK-3β, phospho-GSK-3β and β-actin were detected on total protein extracts, while β-catenin, lamin B and HSP90 detection were performed on nuclear extracts. β-actin and lamin B were used to check for total and nuclear loading, respectively. HSP90 was used to check for separation of nuclear and cytoplasmic fraction. The experiments were performed in duplicate.

**Protein localization analysis.** D283MED and DAOY were cultured on glass coverslips and fixed after 24 h from LiCl addition with 2% paraformaldehyde in PBS, for immunofluorescence (IF) staining of β-catenin as previously described (28). IF was performed using a rabbit polyclonal anti-β-catenin (H-102, Santa Cruz Biotechnology) as primary antibody at the dilution of 1:100 in blocking solution. Primary antibody was detected with a FITC-conjugated swine anti-rabbit antibody (F0205, Dako Cytomation; Glostrup, Denmark) at the dilution of 1:100 in blocking solution. Nuclei were counterstained with ProLong Gold anti-fade Reagent with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen Corporation). The experiments were performed in duplicate.

**Cell proliferation analysis.** D283MED and DAOY were seeded in triplicate in 96-well plates at a density of 5000 and 1000 cells/well, respectively, and maintained in 100 μl of EMEM 20% FBS. The cells were analyzed 24, 48, 72, 96 and 120 h after LiCl addition and at the corresponding time-points for untreated cells. Cell proliferation of both treated and untreated cells was assessed using cell proliferation kit I MTT (Roche Diagnostic GmbH), according to the manufacturer's instructions (number 3.2.1). Resulting absorbance was measured using a microplate reader (Sirio-S, SEAC; Firenze, Italy) as the difference between the formazan product absorbance at a wavelength of 550 nm and the reference absorbance at 660 nm.

**Cell cycle analysis.** In order to evaluate cell cycle changes induced by the treatment, flow cytometric analysis was performed on D283MED and DAOY as previously described (29). We analyzed specific time points: 24, 29, 40, 48 and 72 h after LiCl addition. The experiments were performed in duplicate.

**Cell death analysis.** In order to evaluate cell mortality, D283MED and DAOY were seeded in 25-cm² culture flasks and collected 48 and 72 h after LiCl addition. Untreated cells were harvested at corresponding time-points. Triplicates of each sample were trypsinized and total cells were suspended 1:1 in Trypan Blue solution (Sigma-Aldrich). Dead and total cells of any sample were counted using a Neubauer chamber. For apoptosis analysis, cells were collected 8, 24, 48 and 72 h after LiCl treatment and the percentage of apoptotic cells was measured by flow cytometry, using the Vybrant apoptosis assay kit no. 4 (Molecular Probes, Invitrogen Corporation) according to the manufacturer's instructions. Flow cytometric analyses were performed in triplicate for each sample with a Coulter Epics XL/XL-MCL (Beckman Coulter) equipped with an argon ion laser at 488 nm wavelength and using the System II software.

**Lithium dose assay.** D283MED and DAOY were plated in triplicate in 6-well plates at a density of 50,000 cells/well and, 24 h later, LiCl was added to the growth medium at 0.5, 1, 10 and 20 mM doses. Total D283MED were collected at 1, 2, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, 42, 45, 49 days after the addition of lithium; total DAOY were collected at 1, 2, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, 42, 45, 49, 52, 56, 59, 63, 66, 70 days after LiCl addition. Untreated cells were harvested at corresponding time-points. Triplicates of each sample were counted using a Neubauer chamber, staining total cells 1:1 in Trypan Blue solution (Sigma-Aldrich). After 49 days for D283MED and after 70 days for DAOY the mortality curves in response to LiCl were evaluated.
Statistical analysis. Data were analyzed using the Dunnet’s test and Bonferroni’s multiple comparison test on a graphpad software package for Windows (PRISM5). p<0.05 was considered as statistically significant.

Results

Lithium inactivates GSK-3β in MB cell lines. WB analysis demonstrated the action of lithium in D283MED and DAOY, inhibiting GSK-3β function. In particular, the percentage between the phospho-GSK-3β (inactive) and total GSK-3β share increased after 8, 16, 24, 29, 40, 48 h from LiCl treatment compared to untreated cells in both cell lines (Fig. 1A, B, C and D). Furthermore, levels of nuclear β-catenin were higher than the control in D283MED at 24, 29, 32, 40 and 48 h after LiCl addition, with an evident upregulation at 29 and 40 h from the treatment (Fig. 2A and B). In DAOY, a strong increase in nuclear β-catenin was detectable at 24 h from lithium treatment and at 29, 32, 40 and 48 h protein levels decreased, until they were lower than the ones of untreated cells (Fig. 2C and D). Each sample was checked for separation of nuclear and cytoplasmic fraction by detection of HSP90 (data not shown). IF investigations showed, in D283MED, a widespread internal staining for β-catenin after 24 h of lithium treatment and protein levels were higher than the control (Fig. 3A and B). A clear nuclear accumulation of β-catenin could not be affirmed, due to the particular morphology of these cells because they form cellular aggregates and they have a high nucleus/cytoplasm ratio. In DAOY nuclear β-catenin localization was evident 24 h after LiCl addition (Fig. 3C and D).

Lithium affects cell proliferation, cell cycle and mortality in MB cell lines. After checking that the metabolic activity was directly proportional to the number of cells, as expected, for the two cell lines of this study (data not shown), cell proliferation MTT assay showed that both D283MED and DAOY had a slower proliferation in response to lithium compared to untreated cells. In particular, in D283MED cell proliferation 48-120 h after treatment was statistically significantly lower than cell proliferation of untreated cells (Fig. 4A). In DAOY, we observed a statistically significant metabolic arrest in response to LiCl, from 48 to 120 h of treatment, compared to the control (Fig. 4B). Flow cytometry analysis showed a transient decrease in the S-phase of D283MED at 40 h from LiCl addition and cell cycle was re-established at 48 h of treatment (Fig. 5A). In DAOY, a persistent increase in S-phase was observed 24, 29, 40 and 48 h after LiCl treatment compared to the control. At 72 h, the number of cells in the G2/M phase increased, while cells in the S-phase
decreased slightly compared to previous time-points, in agreement with a cell synchronization in the G2/M phase after escaping a block in the cell cycle (Fig. 5B). In both cell lines, statistically significant cell mortality increased in response to lithium treatment. In particular, in D283MED, the percentages between dead and total cells, respectively, at
48 and 72 h were 16.63, 9.80 for untreated controls and 29.05, 20.17% for treated cells (Fig. 6A). In DAOY, the increase in mortality was greater than in D283MED and the percentages of dead cells were 10.73, 16.24% for untreated cells and 33.05, 39.80% for lithium treatment, at 48 h and 72 h, respectively (Fig. 6B). Increase in cell death was not supported by an increase of apoptotic cells compared to untreated cells, both in D283MED and in DAOY. The amount of apoptotic cells was not statistically significantly different between treated and control samples (Fig. 7A and B).

D283MED die at lithium clinical dose. In D283MED, 20 mM lithium induced a rapid and massive increase in mortality, in fact, after 10 days of treatment, almost all the cells were dead. From the 2nd day of lithium treatment at 20 mM, the increase in mortality of treated cells was statistically significantly higher compared to untreated cells. As regards lithium at 10 mM dose, the first statistically significant increase in mortality was observed from the 10th day of treatment onwards and after 35 days all cells were dead. After treatment with 1 mM lithium, late mortality was induced and a statistically significant increase was observed only from the 42nd day of treatment; then cell mortality further increased. Even if total mortality was not achieved during the observation period, it is important to consider that after 45 days of 1 mM lithium we obtained a mortality comparable to that obtained after 2 days of 20 mM lithium (first time-point statistically significant for 20 mM lithium). In decreasing the
dose of lithium to 0.5 mM, no statistically significant increases in cell mortality were observed (Fig. 8A, Table I). In DAOY, in response to 20 mM lithium we observed a statistically significant induction of mortality from the 2nd day of treatment and after 7 days all of the cells were dead. After the addition of 10 mM lithium, the effect on cell mortality was completely different. We observed statistically significant increases in mortality followed by trend reversals, so the general trend in the observation period is difficult to interpret and after 70 days of treatment the cell population was still mostly alive. Lithium treatment at the clinical doses of 1 and 0.5 mM did not induce cell mortality in DAOY (Fig. 8B, Table II).

Discussion

In the literature, it has been reported that lithium, an inhibitor of GSK-3ß, has anti-neoplastic effects in human melanoma, hepatocellular carcinoma, prostate cancer and glioma (7,12-14). It is well-known that lithium mimics the activation of the Wnt pathway, leading to nuclear accumulation of β-catenin, an independent marker of a good outcome in MB (24-26).

For these reasons, we have analyzed the effects of lithium on two MB cell lines that, deriving from primary tumours of different histopathological variants, have a different molecular arrangement (28,29). In particular, these cell lines are different for the status of p53: one has p53 wild-type and the other harbours mutated p53 (29). In both cell lines, the inhibition of GSK-3ß activity after LiCl treatment was confirmed by the increase of phosphorylated (inactive) GSK-3ß in treated cells compared to untreated. The main action of GSK-3ß is to induce the degradation of β-catenin and, therefore, GSK-3ß inhibition leads to β-catenin stabilization.

Table I. Cell mortality of D283MED in response to different doses of LiCl (mean percentages of dead on total cells).

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p-value (LiCl vs. untreated): *p<0.05; **p<0.01; ***p<0.001.
ß-catenin, the reason why ß-catenin levels decreased to
although we have shown the effectiveness of the drug, in
interaction was revealed by both assays and data are in agreement.
An effect of ß-catenin stabilization due to GSK-3ß inhibi-
ß-catenin localization 24 h after LiCl addition. In all cases,
showed a stronger increase in nuclear ß-catenin. On the other
phenomenon could be observed later in time, when WB data
usually form cellular aggregates, therefore an exact subcel-
are small cells with a high nucleus/cytoplasm ratio and they
after LiCl treatment, as observed by WB. However, D283MED
had constantly higher levels of nuclear ß-catenin
after LiCl treatment, levels of nuclear ß-catenin increased in
proliferation. Even with these small differences, biological
response to lithium was comparable in both cell lines but, as
previously explained, nuclear ß-catenin levels had different
trends. Therefore, we concluded that the observed effects
were completely independent of Wnt-pathway activation; we
cannot definitely exclude that ß-catenin translocation to the
nucleus, even if transient in DAOY, triggered molecular
responses similar to those in D283MED, where high nuclear
levels were persistent, inducing the same cellular effect.
Although it is not possible to clarify the underlying molecular
mechanism with these data, we observed significant biolo-
logical effects in response to 20 mM lithium. The inhibition
of proliferation and the induction of cell death are among the
expected effects of an anti-neoplastic drug. Therefore, we
assessed cell mortality even at doses lower than 20 mM,
including the clinical doses of 1 and 0.5 mM. The clinical
dose of 1 mM lithium had the desired effect of inducing
mortality in D283MED, but not in DAOY, indicating that
lithium could be a promising molecule from a therapeutic
perspective. MB cells are small cells with a high nucleus/cytoplasm ratio and they usually form cellular aggregates, therefore an exact subcellular localization is difficult to evaluate. We observed a widespread internal staining after the treatment, but we did not notice a clear nuclear accumulation. It is possible that this phenomenon could be observed later in time, when WB data showed a stronger increase in nuclear ß-catenin. On the other hand, in DAOY, IF analysis showed an evident nuclear ß-catenin localization 24 h after LiCl addition. In all cases, an effect of ß-catenin stabilization due to GSK-3ß inhibition was revealed by both assays and data are in agreement.

Table II. Cell mortality of DAOY in response to different doses of LiCl (mean percentages of dead on total cells).

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after LiCl treatment, levels of nuclear ß-catenin increased in
both cell lines. Although these data confirmed the action of
lithium downstream of GSK-3ß, it should be noted that
D283MED had constantly higher levels of nuclear ß-catenin
than the control, while DAOY had an increase in nuclear
ß-catenin after 24 h of treatment and then levels decreased
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All these data are in agreement and explain how lithium
induced a decrease in the number of metabolically active
cells. Finally, in DAOY we observed a higher induction of
mortality compared to D283MED and a cell cycle arrest,
explaining the more marked and progressive decrease in
proliferation. Even with these small differences, biological
response to lithium was comparable in both cell lines but, as
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lithium could be a promising molecule from a therapeutic
point of view, depending on the molecular background.

In conclusion, lithium exerts anti-neoplastic effects at the high dose of 20 mM in both in vitro models of MB. From our
data, lithium effects can be assigned to GSK-3 inhibition (32,33), rather than to a specific Wnt activation, even if this molecular pathway is altered by the drug in both cell lines. On the other hand, the clinical dose of 1 mM lithium elicits anti-neoplastic effects only in D283MED cells. Therefore, lithium could be a promising drug in MB, but only in some subtypes of MB with a specific molecular background. Moreover, MB is an extremely heterogeneous tumour, from both a histopathological and a molecular point of view, so it is not probable that a targeted therapy could be effective in all patients. Specific molecular markers predictive of the response of this drug still need to be clarified.

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References

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