Lithium Protects Rat Cerebellar Granule Cells against Apoptosis Induced by Anticonvulsants, Phenytoin and Carbamazepine

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ABSTRACT
We have studied the neuroprotective actions of lithium against various insults in cultured cerebellar granule cells of rats. The anticonvulsants, phenytoin and carbamazepine, have been shown to induce apoptosis of cerebellar granule cells at high concentrations. Here we found that co-presence of LiCl (1–10 mM) dose-dependently protected against phenytoin (20 μM)- and carbamazepine (100 μM)-induced neuronal apoptosis as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide metabolism, morphological inspection, chromatin condensation and DNA fragmentation. These neuroprotective effects were not prevented by inclusion of myoinositol nor mimicked by a potent inositol monophosphatase inhibitor, suggestive of a mechanism independent of inositol monophosphatase blockade. Lithium also significantly protected against apoptosis of cerebellar granule cells induced by aging of the cultures. Additionally, lithium suppressed death of cerebellar granule cells exposed to a low concentration of extracellular potassium. In contrast, it had no protective effect on cell death induced by Ca2+ ionophores, a Na+ channel opener, a protein kinase inhibitor, a nitric oxide donor or H2O2. Thus, lithium has robust neuroprotective effects against apoptotic cell death induced by multiple insults with limited selectivity. These actions provide a new avenue to study the molecular and cellular mechanisms of this drug.

Lithium is best known for its therapeutic efficacy in the treatment of manic depressive illness. Its clinical profile includes the antimanic and antidepressant actions as well as prophylaxis of both mania and depression by reducing the frequency of the bipolar episodes (Schou, 1991; Goodwin and Jamison, 1990; Post et al., 1992). Despite its long-standing clinical use and intensive investigation, there has been no consensus on the molecular mechanisms underlying the therapeutic actions of lithium. Several hypotheses have been presented to explain the mechanism of lithium’s actions. One of the most popular models, the inositol depletion hypothesis, is that acute mania in bipolar illness is caused by hyperactivity of receptor-mediated PI turnover in the brain. Accordingly, lithium, by inhibiting IMPase, would deplete brain inositol levels and dampen PI metabolism (Berridge, 1989). Other modes of lithium’s actions include its regulation of PKC isozymes, G proteins and adenylyl cyclases in several cell types and brain (for review, Manji and Lenox, 1994; Manji et al., 1995). Acute lithium exposure mimics the action of phorbol ester and facilitates several PKC-mediated responses, whereas long-term exposure results in down-regulation of PKC. Chronic lithium administration results in inhibition of the G protein function and attenuation of cyclic AMP generation. Lithium also augments the c-fos mRNA level induced by carbachol, a muscarinic receptor agonist, in PC-12 pheochromocytoma cells (Kalaspudi et al., 1990) and by pilocarpine in rat cerebral cortex (Weiner et al., 1991).

The use of cultured cerebellar granule cells as a model system has advanced our knowledge concerning lithium’s actions. We have found that long-term treatment of these neurons with LiCl at therapeutically related concentrations induces neurotrophic effects and increases the mRNA levels of c-Fos and m3-muscarinic acetylcholine receptors (Gao et al., 1993). More recently, we found that acute and chronic
treatment of cerebellar granule cells with LiCl increases transcription factor binding to AP-1 and CRE consensus DNA sequences (Ozaki and Chuang, 1997). Acute lithium also has been reported to protect these cerebellar neurons against cell death induced by lowering KCl from 25 to 5 mM in the culture medium (D’Mello et al., 1994). This low K+-induced neuronal death involves apoptosis and is suppressed by activation of phospholipase C-coupled m3-muscarinic receptors (Yan et al., 1995a).

Apoptosis, also referred to as programmed cell death, is a process by which a cell dies through activation of intrinsic cell mechanisms that typically require de novo RNA and protein synthesis and involve chromatin condensation and internucleosomal DNA cleavage (Martin et al., 1988; Margolis et al., 1994). Compelling evidence has linked neuronal apoptosis to neurodegenerative diseases such as Huntington’s chorea, Parkinsonism, Alzheimer’s disease and HIV-1 infection-related dementia (Portera-Cailliau et al., 1995; Temlett, 1996; Barinaga, 1993; Yoshioka et al., 1995). It is also possible that apoptosis is involved in the pathogenesis of certain neuropsychiatric disorders such as schizophrenia, because neuronal loss has been found in multiple brain areas in schizophrenic postmortem brain (Margolis et al., 1994). Further, cell death in schizophrenic brains shows no evidence of inflammation or infiltration by lymphocytes or macrophages, consistent with the characteristics of apoptosis (Margolis et al., 1994).

Two anticonvulsants, carbamazepine and phenytoin, have been shown to induce apoptosis of cerebellar granule cells at doses higher than their therapeutic levels (Gao et al., 1995; Saunders et al., 1995; Yan et al., 1995b). In carbamazepine, the apoptosis is blocked by NMDA (a glutamate receptor subtype agonist), cycloheximide (a protein synthesis inhibitor) and aurintricarboxylic acid (a DNase inhibitor) (Gao et al., 1995). Combined treatment with carbamazepine and lithium often potentiates the therapeutical efficacy elicited by either drug alone against bipolar depressive illness (Post et al., 1992), which suggests interactions between these two drugs. Here we examined the effects of lithium on carbamazepine-induced apoptosis of cerebellar granule cells. We also studied whether lithium protects against neurotoxicity elicited by phenytoin and other insults, and explored the potential mechanisms involved.

Materials and Methods

Materials. Carbamazepine was obtained from Research Biochemicals International (Natick, MA). BME, FCS, gentamicin, proteinase K, ara-geno and DNA ladder standards were obtained from Gibco-BRL Life Technologies (Gaithersburg, MD). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell cultures. Cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rats (Taconic Farms, Germantown, NY) and cultured as described previously (Gao et al., 1995). Cerebella were chopped into 400-μm cubes, and the cells were dissociated by trypsinization followed by DNase treatment. The dissociated cells were suspended in BME containing 10% FCS, 2 mM glutamine, 50 μg/ml of gentamicin and 25 mM KCl. The cells were seeded at a density of 2.7×10^6 cells/cm^2 in 24-well plates, 96-well plates, 60-mm Petri dishes or 100-mm Petri dishes (Nunc, Roskilde, Denmark) precoated with poly-L-lysine, depending on the purpose of the experiments. The cells were maintained at 37°C in the presence of 6% CO₂ in a humidified incubator. Cytosine arabinofuranoside (10 μM) was added to the cultures approximately 24 h after plating to arrest the growth of non-neuronal cells. The culture medium was not changed until the cerebellar granule cell cultures were used to avoid neurotoxicity elicited by trace amounts of glutamate present in fresh medium (Schramm et al., 1990). For most studies, the cultures were used on the 7th to 8th DIV; at this time, the cells had matured and differentiated into glutamatergic neurons with a purity of more than 95% (Levi et al., 1988). In routine experiments, cells were treated with 20 μM phenytoin or 100 μM carbamazepine in the absence or presence of indicated concentration of LiCl in culture medium for 72 h before measurements of cell viability.

Measurement of neurotoxicity. The mitochondrial dehydrogenase activity that cleaves MTT was used to determine cell survival in a quantitative colorimetric assay (Mosmann, 1983). The tetrazolium ring of MTT is cleaved by active dehydrogenase enzymes in viable mitochondria, forming a blue colored precipitate, formazan. Cerebellar granule cells were incubated with MTT (125 μg/ml) in the growth medium for 1 h at 37°C. The medium was then aspirated, and the formazan formed was dissolved in dimethyl sulfoxide and quantified spectrophotometrically at 540 nm.

Analysis of DNA fragmentation. DNA fragmentation was assessed with a soluble DNA preparation based on a method described previously (Gao et al., 1995), with minor modifications. Cerebellar granule cells (2×10^6 cells) grown in 100-mm dishes were washed with ice-cold PBS containing 10 mM EDTA (pH 7.2) and scraped off the dish in the same buffer. The cells were collected by centrifugation at 1,000×g for 5 min and the cell pellet was lysed in 250 μl of a buffer consisting of 10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After 15 min on ice, the lysate was centrifuged at 12,000×g for 10 min. The supernatant (containing RNA and fragmented DNA, but not intact chromatin) was treated with proteinase K (0.3 mg/ml) and RNase A (0.3 mg/ml) for 30 min at 37°C and then extracted with NaCl as described (Wang et al., 1994). The DNA was precipitated with 1 volume of isopropanol and centrifuged. The pellet was washed with 70% ethanol, air-dried and dissolved in 10 mM Tris-HCl containing 1 mM EDTA (pH 8.0). The DNA was electrophoresed in a 1.5% agarose gel in TBE buffer and the DNA band was visualized by ethidium bromide staining and photographed.

Detection of chromatin condensation. Chromatin condensation was detected by nucleus staining with Hoechst 33258 as described previously (Gao et al., 1995). Cerebellar granule cells (2.5×10^6 cells) grown on a 35-mm dish were washed with ice-cold PBS, fixed with 4% formaldehyde in PBS for 10 min at 4°C and washed with PBS. Cells were then stained with Hoechst 33258 (5 μg/ml) for 5 min at 4°C, followed by washing with PBS. Stained nuclei were visualized with a Zeiss Axioskop fluorescence microscope at a 1000× magnification with an excitation wavelength of 355 and an emission wavelength of 465 to 480 nm.

Statistical analysis. Data are presented as the mean±S.E.M. from independent experiments. Statistical analysis of data was performed by Student’s t test or one-way repeated measures ANOVA followed by Bonferroni multiple comparison tests.

Results

Lithium protects against neuronal death induced by anticonvulsants, phenytoin and carbamazepine. Exposure of cerebellar granule cells on 7–8 DIV to either phenytoin (1–50 μM) or carbamazepine (10–200 μM) in the medium for 3 days resulted in a concentration-dependent decrease in neuronal viability assessed by the measurement of mitochondrial activity (MTT assay). Cotreatment with 5 mM LiCl markedly reduced neurotoxicity induced by phenytoin and carbamazepine (fig. 1, A and B), whereas exposure to LiCl alone did not affect neuronal viability significantly (results
not shown). The presence of NMDA (100 μM) inhibited carbamazepine-induced neurotoxicity (fig. 1B), which confirmed our previous report (Gao and Chuang, 1992). NMDA also effectively attenuated phenytoin toxicity (fig. 1A). A small but significant increase in the viability of untreated cells was induced by NMDA, which suggests a low degree of spontaneous cell death that also was protected by NMDA. The neuroprotective effects of lithium against phenytoin (20 μM)- and carbamazepine (100 μM)-induced neurotoxicity were dose-dependent in the concentration range of 0.1 to 10 mM tested (fig. 1, C and D). Significant protection against anticonvulsant neurotoxicity was observed at 1 mM LiCl, and maximal effects were found at 10 and 5 mM LiCl for phenytoin and carbamazepine, respectively. The loss of cell viability elicited by either phenytoin or carbamazepine was enhanced progressively by an increase in the treatment time from 24 to 96 h (fig. 1, E and F). The presence of 5 mM LiCl markedly protected against neurotoxicity induced by either drug at all the time points examined.

Morphological examination of viable cells labeled with...
MTT by phase-contrast microscopy revealed that phenytoin or carbamazepine neurotoxicity is associated with the typical appearance of dead cells, whose bodies were round, smaller and translucent, and shared a marked disintegration along with neuronal processes (fig. 2, C and E). Cotreatment with lithium (5 mM) effectively prevented the phenytoin- and carbamazepine-induced morphological changes and increased the numbers of viable cells in these treated cultures (fig. 2, D and F).

Staining the nuclear chromatin with the fluorescent dye Hoechst 33258 revealed the appearance of ‘brightened’ and fragmented nuclei after phenytoin and carbamazepine treatment (fig. 3). This suggests that condensation of nuclear chromatin and disintegration of nuclei had occurred and is characteristic of apoptosis. These anticonvulsant-induced apoptotic bodies were suppressed markedly by the presence of LiCl.

Given that cerebellar granule cell death induced by phenytoin and carbamazepine displays internucleosomal DNA fragmentation (Gao et al., 1995), a hallmark of apoptosis, we examined whether lithium affects this aspect of biochemical change. Exposure of cerebellar granule cells to phenytoin or carbamazepine for 48 h resulted in marked DNA fragmentation revealed as laddering on a gel, and the presence of LiCl (5 mM) prevented the appearance of this DNA laddering almost completely (fig. 4). The presence of LiCl alone had little, if any, effect on DNA laddering.

Myoinositol does not affect and an inositol monophosphatase inhibitor does not mimic lithium neuroprotection. Several in vitro effects of lithium have been attributed to inositol depletion, resulting from inhibition by lithium of IMPase, and are therefore reversible by addition of excessive inositol (Berridge et al., 1989; Brami et al., 1993). However, the presence of myoinositol (1–10 mM) during lithium treatment failed to abolish lithium’s neuroprotection (fig. 5A), which indicates that these effects are unrelated to the reduction of endogenous inositol level. To test further the role of IMPase in lithium’s neuroprotection, we used a novel competitive inhibitor of IMPase, the bisphosphonate L-690,330, which is 1000-fold more potent than lithium in inhibiting IMPase with an IC_{50} value of about 1 μM (Atack et al., 1993; Klein and Melton, 1996). Treatment with L-690,330 in the concentration range of 1 to 100 μM did not protect against phenytoin-induced neuronal death (fig. 5B). In fact, higher concentrations of this IMPase inhibitor produced marked neurotoxicity in both untreated and phenytoin-treated cells. Thus, it seems unlikely that IMPase blockade contributes to the neuroprotective effect of lithium against phenytoin-induced apoptosis.

Lithium inhibits age-induced and low [K^+]_o-induced apoptosis. To investigate whether the effect of lithium is limited to anticonvulsant-induced apoptosis, we tested the involvement of lithium on apoptosis of cerebellar granule cells induced by aging of the cultures. Under typical culture...
conditions, cerebellar granule cells die abruptly after 15–16 DIV. This burst of neuronal death requires de novo RNA and protein synthesis and involves ultrastructural changes and internucleosomal DNA cleavages characteristic of apoptosis (Ishitani et al., 1996). This phenomenon is termed age-induced apoptosis in cultures (Ishitani et al., 1996). When cerebellar granule cells were pretreated with LiCl on the 6 or 8 DIV, age-induced apoptosis was diminished significantly in a concentration-dependent manner. The protection was significant at 1 mM, reached a maximum at 5 mM and was reversed to the control at 10 mM. Pretreatment with LiCl on the 11th or 13th DIV revealed no neuroprotective effect. Thus, lithium protection against age-induced apoptosis depends on long-term preexposure to this drug (fig. 6).

The survival of mature cerebellar granule cells requires the presence of a high concentration of extracellular potassium ([K+]o), i.e., 25 mM, in the culture medium. Confirming the results of D’Mello et al. (1994), we found that switching from high to low [K+]o (15 mM) in the medium of cerebellar granule cells at 7 DIV resulted in a 40% loss of viability within 24 h; this cell loss was markedly protected when LiCl was present during medium change (fig. 7). Additionally, cycloheximide protected this form of apoptosis as well as phenytoin-induced apoptosis. However, neither lithium nor cycloheximide was able to protect against neurotoxicity induced by Ca++ ionophores (A23187 and ionomycin), veratridine (a Na+ channel opener), staurosporine (a protein kinase inhibitor), SNP (a nitric oxide donor) or H2O2 (fig. 7). It is likely that the neuroprotective action of lithium is selective for certain forms of apoptosis that are inhibited by a protein synthesis inhibitor, such as those induced by anticonvulsants, aging of the cultures and deprivation of high [K+]o in the medium.

**Discussion**

In this study, based on assessments by MTT assays, morphological inspection, chromatin condensation and DNA fragmentation results, we found that in a concentration-dependent manner lithium effectively protects cerebellar granule cells against apoptosis induced by two anticonvulsants, phenytoin and carbamazepine (figs. 1–4). Although lithium protection against anticonvulsant-induced apoptosis can occur at the therapeutic concentration of 1 mM, more robust effects are found at $\approx$5 mM with an EC50 value of approximately 3 to 4 mM. Because the protection does not require pretreatment and needs relatively high concentrations of lithium, it is unclear whether this effect is related to its clinical efficacy. It is conceivable, however, that prolonged pretreatment time and/or other experimental manipulations will bring the lithium dose closer to its therapeutic serum level, i.e., 0.5 to 1.5 mM. The protection by lithium against carbamazepine-induced apoptosis may be clinically relevant, because carbamazepine is an alternative drug used for the treatment of bipolar depressive illness and this clinical efficacy is potentiated by coadministration with lithium carbonate (Post et al., 1992). Supratherapeutic doses of carbamazepine can be teratogenic and induce ataxia, nystagmus.
and vertigo, suggestive of cerebellar impairment. If neuronal apoptosis induced by high doses of carbamazepine occurs in vivo and is suppressed by coadministration with lithium, the latter may increase the beneficial effects of carbamazepine by increasing this drug’s efficacy/toxicity ratio.

Confirming a previous result (D’Mello et al., 1994), we found that lithium protects cerebellar granule cells against low $[K^+]_o$-induced apoptosis (fig. 7). Moreover, apoptosis of cerebellar neurons induced by aging of the cultures is reduced effectively by lithium (fig. 6). Unlike the protective effects against anticonvulsant-induced cell death, lithium protection against age-induced apoptosis requires at least 1 week of pretreatment and needs relatively low concentrations of this drug. These results suggest that distinct mechanisms may be involved in lithium-induced protection against age- and anticonvulsant-induced apoptotic processes. We have shown that age-induced apoptosis of cerebellar granule cells is suppressed effectively by NMDA receptor antagonists (Lin et al., 1997) but accelerated by exogenous NMDA (Ishitani et al., 1996), which suggests that the apoptotic process is triggered by overstimulation of NMDA receptors via glutamate endogenously released from cerebellar granule cells under depolarizing culturing conditions. In this context, we reported recently that chronic (7-day) but not acute (1-day) treatment of cerebellar granule cells with LiCl (2 mM) robustly inhibits cell death induced by addition of glutamate (100 $\mu$M) to the culture medium through a mechanism involving inhibition of NMDA receptor-mediated calcium influx (Nonaka et al., 1998). In these studies, glutamate induces primarily neuronal apoptosis, as evidenced by the occurrence of chromatin condensation and internucleosomal DNA cleavage, and lithium apparently protects only the apoptotic but not necrotic component (Nonaka et al., 1998). It should be noted that acute lithium treatment fails to protect against cell death induced by calcium ionophores, a sodium channel agonist, a protein kinase inhibitor, a nitric oxide donor or $H_2O_2$ (fig. 7). Cycloheximide also does not protect against these insults, which suggests that de novo protein synthesis is not involved. It is possible that these neurotoxic effects are largely caused by necrosis, rather than apoptosis, and are therefore resistant to lithium protection. The neuroprotective action of lithium is not restricted to cerebellar granule cells. It has been reported that lithium induces short-term survival of PC12 cells after serum and NGF deprivation (Volonte and Rukenstein, 1993), and survival of GABAergic neurons in the cerebellum and cerebral cortex (Volonte et al., 1994). Lithium also protects PC12 and human SY5Y cells against ouabain-induced neurotoxicity (Li et al., 1994). Additionally, Inouye et al. (1995) reported that lithium delays radiation-induced apoptosis in external granule cells of mouse cerebellum. These observations suggest that lithium has neuroprotective effects against apoptosis induced by a wide variety of stimuli in multiple neuronal cell types.

We have attempted to elucidate the molecular mechanisms underlying lithium-induced neuroprotection against anticonvulsant-induced apoptosis. One of the best known intracellular effects of lithium is inhibition of IMPase, which causes accumulation of inositol phosphates and depletion of inositol (Berridge et al., 1989). In fact, it has been reported that lithium modulates PI turnover in cerebellar granule cells (del Rio et al., 1996). However, we found that supplement of excess myoinositol does not affect lithium’s protective effect, and a potent IMPase inhibitor, L-690,330, fails to mimic the protective action (fig. 5). Thus, the neuroprotective mechanism apparently is independent of inhibition of PI turnover by blocking IMPase. Lithium increases transcription factor

![Fig. 4. Lithium inhibits phenytoin- and carbamazepine-induced internucleosomal DNA fragmentation of cerebellar granule cells. Cells were exposed for 48 h to phenytoin (PHT, 20 $\mu$M) or carbamazepine (CBZ, 100 $\mu$M) in the absence or presence of LiCl (5 mM). Soluble DNA was prepared from cell extracts and subjected to agarose gel electrophoresis, stained with ethidium bromide and photographed as described under “Methods.” Data are from a representative experiment repeated three times with similar results.](image-url)
binding to AP-1 and CRE consensus DNA sequences and increases the mRNA levels of c-Fos and m3-muscarinic receptors in cerebellar granule cells (Gao et al., 1993; Ozaki and Chuang, 1997). Therefore, the neuroprotective effects could be the consequence of lithium-induced gene expression of neuroprotective proteins.

In low [K^+]_o-induced apoptosis of cerebellar granule cells, the neurotoxicity is suppressed by either LiCl (D’Mello et al., 1994) or activation of m3-muscarinic receptors (Yan et al., 1995a), which raises the possibility that the neuroprotection against anticonvulsant neurotoxicity could be related to induction or activation of m3-muscarinic receptors as reported in our previous study (Gao et al., 1993). This hypothesis also can be ruled out because lithium protection is unaffected by the presence of a muscarinic receptor antagonist, atropine (data not shown). Both carbamazepine and phenytoin inhibit NMDA receptor-mediated calcium influx in cerebellar granule cells (Hough et al., 1996). Neurotoxicity elicited by carbamazepine and phenytoin can be suppressed effectively by the co-presence of NNMDA (fig. 1, Gao and Chuang, 1992; Gao et al., 1995). Thus, it could be argued that lithium-induced neuroprotection is mediated though activation of NMDA receptors. However, our observations that NMDA receptor antagonists, such as MK-801 and 2-amino-5-phosphono-pentanoate, fail to affect lithium protection (data not shown) speak against this possibility. Taken together, our results strongly suggest that lithium protection against anticonvulsant-induced apoptosis is unrelated to IMPase blockade and activation of muscarinic or NMDA receptors.
Recently, it was shown that lithium is a potent and selective inhibitor of glycogen synthase kinase-3β, a highly conserved serine/threonine kinase implicated in cell fate determination during development (Siegfried et al., 1990; Klein and Melton, 1996; Pytle et al., 1992). Insulin and growth factors also inhibit this kinase through activation of the protooncogenic serine/threonine kinase, Akt, by receptor tyrosine kinase-mediated stimulation of PI-3 kinase (D’Mello, 1997). Activation of PI-3 kinase was shown previously to be required for NGF-mediated survival in the PC 12 cell line (Yao and Cooper, 1995). Moreover, Calissano et al. (1995) showed that the serum enhancement of cerebellar neuron survival was inhibited partly by anti-IGF-I monoclonal antibodies, which suggests that the serum-survival effect is mediated, at least in part, through IGF-I or IGF-I-like activity. Furthermore, IGF-I appears to protect against low [K+]o-induced neuronal apoptosis through activation of PI-3 kinase (D’Mello et al., 1997). Collectively, lithium might mimic the effects of NGF and IGF-I through activation of PI-3 kinase and inhibition of glycogen synthase kinase-3β in inducing neuroprotection against anticonvulsant-induced apoptosis.

Alternatively, lithium might exert its protective effects by modulating the level of intracellular calcium concentration ([Ca2+]o). Phenytin reduces basal [Ca2+]o in cerebellar granule cells (Yan et al., 1995b), presumably through inhibition of NMDA receptor activation by endogenous glutamate released into the culture medium (Hough et al., 1996). It is conceivable that carbamazepine and low [K+]o also reduce basal [Ca2+]o by blocking Ca2+ influx mediated through NMDA receptors and voltage-sensitive calcium channels, respectively. Thus, an [Ca2+]o insufficient to promote cell survival could be the molecular basis of anticonvulsant and low [K+]o-induced apoptosis; and lithium, by an undefined mechanism, may restore the loss of [Ca2+]o, induced by these insults. In this context, it is noteworthy that lithium stimulates the release of glutamate from cerebrocortical slices (Hokin et al., 1996). Experiments are in progress to test all these possibilities. Although the molecular mechanisms underlying the neuroprotective activities of lithium are still unclear, our studies have moved an important step toward further understanding of lithium’s cellular effects and have provided a new avenue to investigate their potential relevance to the clinical actions of this drug.

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References


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