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Mini-Review

Citicoline Mechanisms and Clinical Efficacy in Cerebral Ischemia

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Citicoline, an intermediate in the biosynthesis of phosphatidylcholine (PtdCho), has shown beneficial effects in various CNS injury models and neurodegenerative diseases. PtdCho hydrolysis by phospholipase A_2 (PLA_2) after cerebral ischemia and reperfusion yields arachidonic acid (ArAc) and lyso-PtdCho. ArAc oxidative metabolism results in formation of reactive oxygen species and lipid peroxides. Lyso-PtdCho could inhibit activity of cytidine triphosphate-phosphocholine cytidylyltransferase (the rate-limiting enzyme in PtdCho biosynthesis), resulting in impaired PtdCho synthesis. Citicoline significantly increased glutathione levels and attenuated release of ArAc and the loss of PtdCho, cardiolipin, and sphingomyelin following transient cerebral ischemia. These effects could be explained by an effect of citicoline on PLA_2. Based on these observations, a mechanism has been hypothesized. This Mini-Review summarizes recent experimental data on the effects of citicoline in cerebral ischemia and evaluates several factors that might have hindered efficacy of citicoline in stroke clinical trials in the United States. Clinical stroke trials of citicoline in Europe and Japan have demonstrated beneficial effects. U.S. trials shown only marginal effects, which might be due to the 24 hr time window, the dose and route of administration, and the stringency of the primary outcome parameters. Recent evaluation of U.S. clinical data suggests that reduction of infarct growth may be a more sensitive measure of the citicoline effect than improvement on the NIH Stroke Scale (NIHSS) by $\geq$7 points. The citicoline neuroprotective mechanism has not been clearly identified, and its potential in stroke treatment might still be fully recognized in the United States. The clinical efficacy of citicoline should be examined further in light of the recent phase III stroke clinical trials and experimental data for cerebral ischemia.

Key words: cardiolipin; glutathione; lipid peroxidation; mitochondria; neuroprotection; phosphatidylcholine; phospholipase A_2; phospholipids; reactive oxygen species; sphingomyelin

CITICOLINE NEUROPROTECTION IN TRANSIENT CEREBRAL ISCHEMIA

One of the common features of cerebral ischemia, whether global or focal, is energy failure and activation of phospholipases (Siesjo, 1992a,b). Because the process of neuronal death continues for several days or possibly for weeks (for example, transient forebrain ischemia in the gerbil; see Fig. 1), there exists an opportunity for therapeutic intervention in the sequence of molecular events following transient cerebral ischemia. Several studies have been conducted with citicoline in stroke models, showing a decrease in ischemic injury volume or improvement in behavioral parameters (D'Orlando and Sandage, 1995; Schabitz et al., 1996, 1999; Shuaib et al., 2000), but mechanistic data were not presented. A recent study (Krupinski et al., 2002) showed that citicoline reduced the expression of procaspases 1, 2, 3, 6, and 8 in the infarct region, as well as DNA fragmentation and expression of active caspase-3, in the penumbra region after permanent middle cerebral artery occlusion (MCAO) in rat. Poly (ADP-ribose) polymerase (PARP, 89 kDa) expression was also reduced in citicoline treated ischemic rats. These results suggested that citicoline inhibits the expression of proteins involved in apoptotic cascade following MCAO. These effects were attributed to effects of citicoline on PtdCho metabolism, although no experimental evidence was presented. Additionally, these results were not correlated with neuroprotection since the effect of citicoline on ischemic injury volume was not determined due to large variations observed within the treatment groups.

Phosphatidylcholine (PtdCho) is the major membrane phospholipid in mammalian cells. In addition to

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being a principal structural component of cellular membranes, PtdCho can be utilized as the biosynthetic precursor for other phospholipids such as sphingomyelin and phosphatidylserine (PtdSer), serves as a reservoir for several lipid messengers, and is the source of bioactive lipids such as phosphatidates, 1,2-diacylglycerol (DAG) and arachidonic acid (ArAc). Citicoline neuroprotection is considered to be due to increased phosphatidylcholine (PtdCho) synthesis in the injured brain; however, in our studies, citicoline only partially restored PtdCho, whereas sphingomyelin and cardiolipin were completely preserved (Rao et al., 2000, 2001). Citicoline also had beneficial effects in various CNS injuries and neurodegenerative diseases, including transient forebrain ischemia (Rao et al., 1999a,b, 2000; Grieb et al., 2001). This suggests a common underlying mechanism for citicoline associated with stabilization or repair (Aronowski et al., 1996; Adibhatla et al., 2002a) of damaged cellular membranes (Wattiaux-De Coninck and Wattiaux, 1994). A recent review also provided a rationale for development of citicoline in glaucoma treatment (Grieb and Rejdak, 2002). The present Mini-Review summarizes recent experimental data on the effects of citicoline in cerebral ischemia and highlights certain factors that might have affected the efficacy of citicoline in stroke clinical trials in the United States.

CITICOLINE METABOLISM

Citicoline is composed of cytidine and choline linked by a diphosphate bridge. In rodents, exogenous citicoline is hydrolyzed and absorbed as cytidine and choline (Secades and Frontera, 1995; Weiss, 1995; Wurtman et al., 2000). After absorption, choline and cytidine are rephosphorylated, and citicoline must be resynthesized from cytidine triphosphate and phosphocholine (choline monophosphate) by cytidine triphosphate-phosphocholine cytidylyl transferase (PCCT; Kent, 1997). PtdCho is synthesized from citicoline and 1,2-diacylglycerol (DAG) by citicoline:DAG choline phosphotransferase (CPT; McMaster and Bell, 1997):

$$\text{cytidine triphosphate} + \text{phosphocholine} \xrightarrow{\text{PCCT}} \text{citicoline} + \text{phosphate}$$

$$\text{citicoline} + \text{DAG} \xrightarrow{\text{CPT}} \text{PtdCho} + \text{cytidinemonophosphate.}$$

Because citicoline is normally the rate-limiting intermediate in PtdCho biosynthesis, it was believed that citicoline administration would provide benefit in pathological conditions, such as CNS injury, in which membrane damage contributes to neuronal death (Wattiaux-De Coninck and Wattiaux, 1994). DAG levels also have been shown to be rate-limiting when PC12 cells are stimulated (Araki and Wurtman, 1997, 1998). However, our studies show that DAG levels were unaltered over 6 days after transient ischemia, with or without citicoline treatment (Adibhatla et al., 2001). In vitro studies have shown that cytidine and choline, the circulating metabolites of citicoline in rodents, increased both the rate of synthesis and the total amount of PtdCho in PC12 cells and rat striatal slices (Lopez-Coviella and Wurtman, 1992; Savci and Wurtman, 1995). In contrast, in vivo studies demonstrated an increase in brain PtdCho levels in normal female Sprague-Dawley rats only after 42 days of citicoline (500 mg/kg/day) and not after 21 days (Lopez-Coviella et al., 1995). Thus, there were differences between cell culture and in vivo studies using citicoline, and results from in vitro systems may not translate into the in vivo or clinical setting.

PHOSPHOLIPID STATUS AFTER CEREBRAL ISCHEMIA

To the best of our knowledge, there are no data on the effects of citicoline on phospholipids in focal cerebral ischemia. One study of permanent forebrain ischemia in the gerbil showed a significant decrease in PtdCho (Trovarelli et al., 1981). Intracerebroventricular pretreatment with citicoline partially restored PtdCho levels and decreased the release of free fatty acids. This was attributed to stimulation of the choline phosphotransferase reaction to increase incorporation of DAG into PtdCho. In these studies, cerebral levels of the drug were likely to be much higher than when the drug is administered systemically. In our studies, systemic pretreatment with citicoline (i.p.) did not significantly alter free fatty acid levels after 10 min of permanent ischemia (no reperfusion; Rao et al., 1999a).

Transient forebrain ischemia resulted in significant decreases in major phospholipids, except for phosphatidylethanolamine (PtdEtn; Rao et al., 2000). Citicoline completely preserved cardiolipin and sphingomyelin; significantly but partially restored PtdCho; and had no effect on PtdEtn, phosphatidylinositol (PtdIns), or phosphatidylserine. PtdCho can be hydrolyzed by phospholipase A_2 (PLA_2), PtdCho-phospholipase C (PLC), and phospholipase D (PLD; Li et al., 1998). The loss of PtdIns indicates activation of a PtdIns-specific PLC (Rhee and Bae, 1997).

Citicoline treatment significantly attenuated arachidonic acid (ArAc) release after transient forebrain ischemia (Rao et al., 1999a, 2000). PLA_2 hydrolyzes ArAc at the *sn*-2 position of PtdCho, and to a lesser extent PtdEtn (Dennis, 1997; Farooqui et al., 2000b). Na^+/K^+-ATPase activity is inhibited by ArAc (Chan and Fishman, 1984) and was restored by citicoline (Rigoulet et al., 1979). These observations suggest an effect of citicoline on PLA_2 activation and the subsequent ArAc release. The observations that citicoline did not restore PtdIns and only partially restored PtdCho levels suggest that it had no effect on activities of phosphoinositide (PI)-PLC, PtdCho-PLC, or PtdCho-PLD (Fig. 2; Rao et al., 2001). However, there is currently no direct data available on the effects of citicoline on these phospholipases.

There is substantial evidence that PLA_2 is activated in ischemia/reperfusion and contributes to neuronal damage (Bonventre et al., 1997; Lipton, 1999; Farooqui et al.,

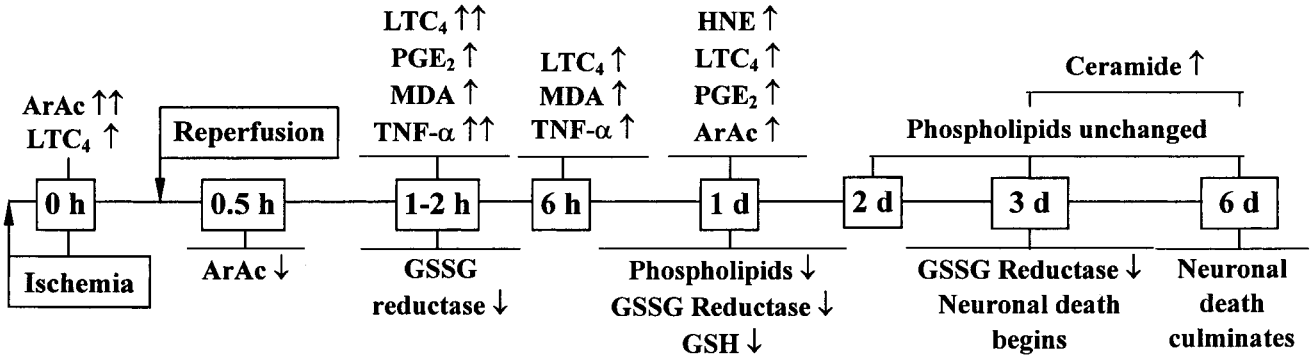

Fig. 1. Time course of biochemical and histological changes in the hippocampus after transient forebrain ischemia in gerbil (Saito et al., 1996; Rao et al., 1999a,b, 2000; Adibhatla et al., 2001). GSSG, glutathione (oxidized); HNE, 4-hydroxynonenal; LTC_4, leukotriene C_4; MDA, malondialdehyde; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-α. ↑, Significant increase; ↑↑, maximal increase; ↓, significant decrease compared with shams.

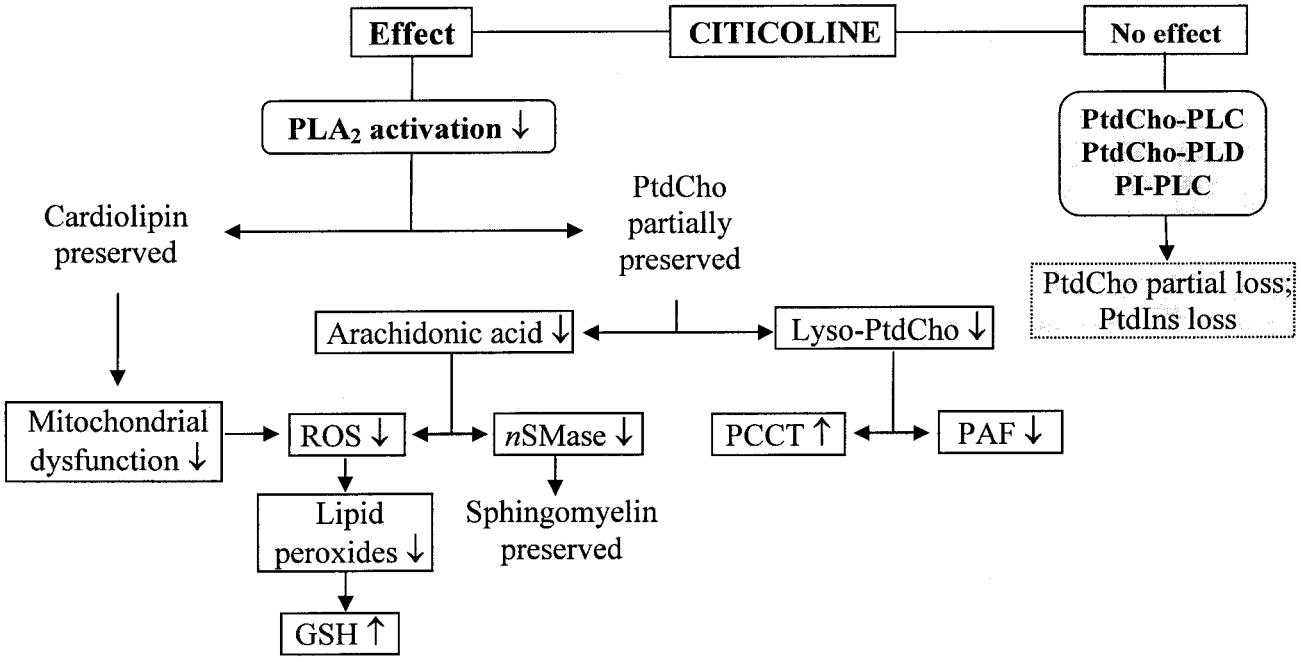

Fig. 2. Proposed mechanism of citicoline action. nSMase, neutral sphingomyelinase; PAF; platelet-activating factor; PI; phosphoinositide. The arrows within the text boxes indicate a decrease (↓) or increase (↑).

2000a). However, only one study (Arrigoni et al., 1987) directly examined the effect of citicoline on PLA_2. Citicoline prevented the increase in mitochondrial PLA_2 activity following cryogenic brain injury in rabbit, in which energy failure does not occur as it does in focal or global cerebral ischemia. It was concluded that citicoline prevented PLA_2 activation, because citicoline had no effect on PLA_2 activity in noninjured controls and thus as such is not a "direct PLA_2 inhibitor."

Sphingomyelin hydrolysis by sphingomyelinase (SMase) produces ceramide and results in redistribution of cholesterol and/or enhanced efflux of cholesterol, causing major changes in membrane structural order and integrity (Tepper et al., 2000). Citicoline completely restored sphingomyelin levels after ischemia/1 day of reperfusion. Sphingomyelin can be synthesized using either PtdCho or citicoline as the phosphocholine donor to ceramide (Rao et al., 2000). Activation of neutral SMase may be mediated through PLA_2 and release of ArAc (Jayadev et al., 1994). Citicoline could restore sphingomyelin either by increasing synthesis or by modulating activation of SMase through an effect on PLA_2 activity.

Cardiolipin is an exclusive inner mitochondrial phospholipid enriched with unsaturated fatty acids and is essential for mitochondrial electron transport (Kirkland et al., 2002). Citicoline prevented the loss of cardiolipin at 1 day of reperfusion. Previous studies indicate that the mitochondrial PLA_2 is a Ca^{2+}-dependent, 14 kDa, group IIA secretory PLA_2 (*s*PLA_2) isoform that acts on PtdCho, PtdEtn, and cardiolipin (Nakahara et al., 1992; Zhang et al., 1999). Citicoline may have prevented cardiolipin hydrolysis by inhibiting the activation of this isoform.

THE PLA_2 PRODUCT Lyso-PtdCho INHIBITS PCCT ACTIVITY

Under normal conditions, PtdCho homeostasis is regulated by the balance between the opposing actions of PCCT and the combined effects of PLA_2, PtdCho-PLC, and PtdCho-PLD (Tronchere et al., 1994; Baburina and Jackowski, 1999). PCCT synthesizes citicoline and is the rate-limiting enzyme in PtdCho formation (Kent, 1997). Lyso-PtdCho inhibits PCCT activity (Awasthi et al., 2001; Boggs et al., 1995), which could result in impaired PtdCho synthesis. Because inhibition of PtdCho synthesis is sufficient in itself to cause cell death (Cui et al., 1996), loss of PtdCho may be an important factor contributing to neuronal injury. Citicoline stimulated PCCT (Gimenez et al., 1999) and could restore its activity after ischemia, which is consistent with an effect on PLA_2.

LIPID PEROXIDATION AND GLUTATHIONE

Reactive oxygen species (ROS) and lipid peroxides have been proposed to be significant mechanisms of tissue damage in ischemia/reperfusion (Chan, 2001; Adibhatla et al., 2002c; Kirkland et al., 2002). Citicoline decreased lipid peroxidation following transient cerebral ischemia (Fresta et al., 1994), suggesting that citicoline neuroprotection may include attenuation of ROS.

Glutathione (GSH) is one of the primary antioxidant defenses in the brain to remove H_2O_2 and lipid peroxides (Cooper and Kristal, 1997). After forebrain ischemia, exogenous citicoline increased total GSH over 1 day of reperfusion and decreased the GSH oxidation ratio, suggesting that citicoline attenuated the oxidative stress (Adibhatla et al., 2001). Citicoline also increased GSH reductase activity and may have prevented inactivation of the enzyme by attenuating ROS formation (Adibhatla et al., 2001).

The effects of citicoline on selective phospholipids would help stabilize the cellular membrane and restore mitochondrial function. It is likely that these effects contribute to citicoline neuroprotection, insofar as loss of phospholipids (Siesjo et al., 1995; Farooqui et al., 1997; Rao et al., 1999b) and generation of ROS (Chan, 2001; Adibhatla et al., 2002c; Kirkland et al., 2002) contribute to ischemic injury. That citicoline had no effect on PtdIns or phosphatidylserine and partially restored PtdCho suggests that its ability to restore membrane integrity may be limited. Most of the effects of citicoline could be explained by an action on PLA_2, and a central mechanism has been proposed (Fig. 2). In that energy failure and phospholipases activation occur both in global and in focal cerebral ischemia, the effects of citicoline on PLA_2 could be applicable to focal cerebral ischemia.

CITICOLINE CLINICAL TRIALS

Stroke is a devastating condition that remains the number one cause of adult disability in the United States and the number three cause of death. Tissue plasminogen activator (tPA) is the only drug that has received FDA approval for stroke treatment. Citicoline in animal studies provided significant neuroprotection (Schabitz et al., 1996; Rao et al., 1999a,b; Grieb et al., 2001). Citicoline has undergone 13 clinical stroke trails, including four in the United States (Clark et al., 1997, 1999, 2001; Warach, 2002). Trials in Europe (Boudouresques and Michel, 1980; Goyas et al., 1980) and Japan (Tazaki et al., 1988) and the initial U.S. trail (Clark et al., 1997) showed significant benefit, although subsequent trails in the United States (Clark et al., 1999, 2001) provided disappointing results. A recent analysis of infarct volumes determined by magnetic resonance imaging (MRI) measurements (Warach, 2001) indicated a significant dose-dependent reduction in infarct growth by citicoline (Warach, 2002). Metaanalyses of seven controlled clinical stroke trials showed that citicoline treatment was associated with significant reductions in rates of long-term death or disability (Saver, 2002). In light of theses recent clinical evaluations and experimental data in cerebral ischemia, the clinical efficacy of citicoline might require further consideration. The following factors might have significantly affected the outcome of the clinical trials (Adibhatla et al., 2002b).

Metabolism of Citicoline in Humans vs. Rodents

Citicoline metabolism in humans differs from that in rodents (Wurtman et al., 2000). In rodents, citicoline administration increases blood plasma levels of cytidine and choline, whereas, in humans, blood plasma levels of uridine but not cytidine are increased because of cytidine deaminase in the gastrointestinal tract and liver (Wurtman et al., 2000). Uridine must then enter the brain, become phosphorylated to uridine triphosphate, and then be converted to cytidine triphosphate to be utilized in resynthesis of citicoline.

Brain Uptake

The major difference between the U.S. and the non-U.S. trials is the route of administration (Clark et al., 2001). Clinical trials outside the United States used i.v. administration, in contrast to the oral route used in U.S. trials. It is generally believed that bioavailability is the same between oral and i.v. routes. This conclusion was based on absorption and excretion, not delivery of citicoline to the brain (Secades and Frontera, 1995). Animal studies have shown brain uptake of citicoline (or its metabolites) of only 0.5% of the oral dose, which increased to ~2% when citicoline was administered i.v. Liposome encapsulation of citicoline could further increase brain uptake of the drug

to 23% of the dose (Fresta et al., 1995). Another related factor may be the dose used in clinical trials (500–2,000 mg/day). Although dose-response data from animal studies cannot be readily extrapolated to humans, citicoline at 500 mg/kg (Schabitz et al., 1996) provided significant benefit, whereas lower doses [100 mg/kg (Schabitz et al., 1996) or 152 mg/kg (Sato et al., 1988)] had no effect.

Window of Opportunity

In the U.S. trials (Clark et al., 1997, 1999, 2001), patients were admitted into the clinical studies up to 24 hr after the onset of symptoms, much longer than is used in typical stroke clinical trials (Devuyst and Bogousslavsky, 2001). The time window (Grotta, 2002) may be another major factor contributing to the marginal effects seen in the U.S. trials. All neuroprotective drugs are less effective the later they are given, and most are ineffective if administered more than 2–4 hr after onset of stroke symptoms (Grotta, 2002). Our experimental studies indicated that citicoline does not provide neuroprotection if the onset of treatment is delayed by 3 hr.

Primary Outcome Measure

The NIH Stroke Scale (NIHSS; a composite score obtained from a 3 point rating scale on 11 items; Wityk et al., 1994) and modified Rankin scale (6 point rating scale of disability; 0 being no symptoms at all and 6 being death; Rankin, 1957) are evaluation instruments for stroke severity widely used in clinical trials and practice to assess neurologic outcome and degree of recovery. The instruments' reliability and validity are well documented in the scientific literature. NIHSS has been used most recently in clinical trials of recombinant tPA (r-tPA) in ischemic stroke.

Pooled MRI data from two clinical trials using 500 and 2,000 mg citicoline/day for 6 weeks with final evaluation at 12 weeks showed a significant dose-dependent reduction in the percentage change in lesion volume (baseline to 12 weeks; Warach, 2002). When the primary clinical outcome measure was improvement by $\geq$7 points on the NIHSS (Clark et al., 2001), citicoline at 2,000 mg/day for 6 weeks did not show significant benefit at 12 weeks. It was suggested that changes in lesion volume may be a more sensitive marker for citicoline effects than improvement on the NIHSS by $\geq$7 points (Warach, 2002). However, these results have to be interpreted with caution, because changes in lesion volume do not necessarily correlate with the behavioral outcome. Rankin return to baseline and NIHSS $\leq$1 showed significant improvement at 6 weeks but not at 12 weeks (Clark et al., 2001). Citicoline is well tolerated, with few side effects, so longer term treatment might have provided more robust improvement at 12 weeks (Clark et al., 2001).

CONCLUSIONS

Given the excellent safety profile of citicoline, its potential in stroke treatment might not yet be fully realized. Because of the multiple pathways involved in ischemic injury, no single agent is likely to provide complete neuroprotection following transient ischemia (White et al., 2000). Combining citicoline with agents acting on different mechanisms of ischemic injury provided synergistic benefits in experimental ischemia models (Onal et al., 1997; Andersen et al., 1999; Schabitz et al., 1999; Shuaib et al., 2000).

Hypothermia, resulting in moderate decreases in brain temperature, either during ischemia or reperfusion, is one of the most potent therapeutic approaches in reducing experimental ischemic brain injury (Colbourne et al., 1997; Schmid-Elsaesser et al., 1999). However, hypothermia alone results in only temporary reduction in ischemic injury but may delay the onset of permanent damage. Thus, using hypothermia to delay the process of ischemic injury may provide a longer time-to-treatment window for intervention with a neuroprotective agent such as citicoline. The citicoline neuroprotective mechanism has not been clearly delineated, and citicoline's potential in stroke treatment might still be fully recognized in the United States. Understanding the mechanism of citicoline neuroprotection and also its limitations might lead to more efficacious applications of this drug in stroke therapy, including more efficient delivery systems.

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