# Cytidine 5′-Diphosphocholine (CDP-Choline) in Stroke and Other CNS Disorders

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## Abstract

Brain phosphatidylcholine (PC) levels are regulated by a balance between synthesis and hydrolysis. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1α/β) activate phospholipase $A_2$ ($PLA_2$) and PC-phospholipase C (PC-PLC) to hydrolyze PC. PC hydrolysis by $PLA_2$ releases free fatty acids including arachidonic acid, and lyso-PC, an inhibitor of CTP-phosphocholine cytidylyltransferase (CCT). Arachidonic acid metabolism by cyclooxygenases/lipoxygenases is a significant source of reactive oxygen species. CDP-choline might increase the PC levels by attenuating $PLA_2$ stimulation and loss of CCT activity. TNF-α also stimulates proteolysis of CCT. TNF-α and IL-1β are induced in brain ischemia and may disrupt PC homeostasis by increasing its hydrolysis (increase $PLA_2$ and PC-PLC activities) and inhibiting its synthesis (decrease CCT activity). The beneficial effects of CDP-choline may result by counteracting TNF-α and/or IL-1 mediated events, integrating cytokine biology and lipid metabolism. Re-evaluation of CDP-choline phase III stroke clinical trial data is encouraging and future trails are warranted. CDP-choline is non-xenobiotic, safe, well tolerated, and can be considered as one of the agents in multi-drug treatment of stroke.

### Keywords

Cerebral ischemia; citicoline; clinical trials; interleukin-1β; phospholipases; phospholipids; reactive oxygen species; tumor necrosis factor; lipidomics

## INTRODUCTION

Stroke or "brain attack" is the first leading cause of long-lasting disability, third leading cause of death and continues to be a problem of vast clinical significance. Approximately 3.9 million Americans are stroke survivors, and the after-effects of stroke require more than $51 billion in healthcare costs annually. Presently, tissue plasminogen activator (tPA) is the only FDA approved drug for the treatment of acute ischemic stroke but needs to be administered within 3 h (1). However, there are some concerns that tPA has neurotoxic side effects in addition to its beneficial (thrombolytic) actions (2). Many neuroprotective agents have undergone phase III clinical trials for stroke; most of the trials were abandoned due to ineffectiveness or toxicity of the drug.

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Cytidine-5′-diphosphocholine (CDP-choline or Citicoline) is composed of cytidine and choline linked by a diphosphate bridge and is an essential intermediate in the synthesis of phosphatidylcholine (PC), a major brain phospholipid, via Kennedy pathway. Exogenous CDP-choline is hydrolyzed and absorbed as cytidine and choline (3), and CDP-choline is re-synthesized from cytidine triphosphate (CTP) and phosphocholine by CTP-phosphocholine cytidylyltransferase (CCT), the rate-limiting enzyme in PC synthesis (see [4] and references therein). CDP-choline also serves as a choline donor in the biosynthesis of the neurotransmitter acetylcholine (5). As the intermediate in PC biosynthesis, it was believed that CDP-choline would rectify membrane damage and provide benefit in CNS disorders and injury (including stroke).

CDP-choline has been studied in >11,000 volunteers and patients and showed beneficial effects in cerebral ischemia, traumatic brain injury, hypoxia, Alzheimer's and Parkinson's diseases, learning and memory disorders, alcoholism, drug addiction, amblyopia and glaucoma (Table I.) Citicoline, an international non-proprietary name of CDP-choline, is marketed as a prescription drug (in Japan, Spain, France and Italy) or as an over-the-counter dietary supplement in USA. CDP-choline was originally developed for stroke treatment by Ferrer Internacional, S. A. (Barcelona, Spain) and is being tested for treatment of Alzheimer's and Parkinson's disorders (7). CDP-choline is the only agent that is non-xenobiotic and has virtually no side effects. In 1983, 22 articles were published that described the physi-co-chemical properties, pharmacokinetics, toxicity and bioavailability of this agent (54). CDP-choline (600 or 1000 mg/day) or placebo to healthy volunteers did not show any abnormal side effects in terms of hematological or clinical analysis (3). No clinically significant ECG and EEG abnormalities were noticed. Neurological tests, tendon reflexes, blood pressure and heart rate were not affected by any dose of the drug or placebo. The tolerance of CDP-choline is excellent and side effects were rare, never severe and consisted mainly of digestive intolerance, gastrointestinal discomfort and restlessness. In no case was it necessary to interrupt the treatment for side effects attributed to CDP-choline use (55). Recent re-evaluation of USA phase III stroke clinical trial data is encouraging (49) and this agent still holds promise for treatment of acute ischemic stroke. Indevus Inc. licensed exclusive North American rights from Ferrer Internacional, S. A. for the manufacture, use and sale of CDP-choline for the treatment of stroke.

## CDP-CHOLINE IN CLINICAL TRIALS

### Stroke

There have been 13 stroke clinical trials of CDP-choline since 1980 (nine in Europe and Japan and four in the USA) (5). The European clinical trials showed that CDP-choline improved global and neurological function and promoted earlier motor and cognitive recovery. A large multi-center study in Japan found that CDP-choline showed improvement in a global outcome rating scale. Four major clinical trials in the USA have provided ambiguous results, and thus the beneficial effects of CDP-choline have not been established (49 and references therein). In the first study, CDP-choline improved functional outcome and reduced neurologic deficit. However, two subsequent studies failed to demonstrate improvement in the outcome. On *post-hoc* analysis, CDP-choline was shown to provide beneficial effects in a subgroup of moderate-to-severe stroke cases. Subsequent pooling of individual patient data from four US trials showed that CDP-choline treatment for 6 weeks improved overall recovery at 12 weeks in acute ischemic stroke patients (49 and references therein). Pooled diffusion-weighted magnetic resonance imaging (DW-MRI) data from two clinical trials showed a significant dose-dependent reduction on percent change in lesion volume (56). We have summarized recent experimental data on the effects of CDP-choline in cerebral ischemia and evaluated several factors which might have hindered efficacy of CDP-choline in stroke clinical trials in the USA. One of the factors is the brain uptake of CDP-choline. The European and Japanese trials used

i.v. administration in contrast to oral route used in the USA trails. In animal studies, brain uptake of CDP-choline or its metabolites was 0.5% of the oral dose, whereas i.v. administration elevated brain uptake to 2%. Liposome encapsulation of the drug can further increase brain uptake up to 23% (8,21) and also circumvent CCT, the key rate-limiting enzyme in PC synthesis. Liposome encapsulation suggests a possible strategy to increase the CDP-choline levels in the CNS and enhance its clinical effectiveness (8). In the light of recent clinical evaluations, experimental data in cerebral ischemia, and realization that oral administration was not appropriate, new phase III stroke clinical trials are warranted. Benefit from CDP-choline in humans is far from proven, however future trials are essential before making any conclusions that CDP-choline is ineffective for stroke treatment.

### Alzheimer's Disease and Other Memory Disorders

Clinical studies have demonstrated that CDP-choline improves cognitive performance in elderly subjects (36). Blocking synthesis of PC is sufficient in itself to cause cell death (57, 58), and a 10% loss of cellular membrane is a threatening situation for neuronal viability (59). Alzheimer's brains have shown loss of PC and phosphatidylethanolamine (60) and CDP-choline may rectify the membrane damage in these brains (36). Additionally, the cholinergic system is dysfunctional in Alzheimer's brain (50), and CDP-choline may provide benefit by enhancing acetylcholine synthesis (Fig. 1A).

### Parkinson's Disease

CDP-choline stimulates tyrosine hydroxylase activity and dopamine release (3), which may be due to increases in brain acetylcholine since choline administration produced the same effects (61). Parkinson's disease is characterized by a selective degeneration of the dopaminergic neurons of the substantia nigra (62), however the phospholipid abnormalities present in Alzheimer's brains were not observed (60). Levodopa is the main therapeutic option for treatment of Parkinson's disease; its main disadvantage is progressive loss of efficacy (63). CDP-choline has been tested in treatment of Parkinson's disease because of its ability to increase the availability of dopamine (3). Combination treatment of Parkinson's patients with CDP-choline and levodopa allowed significant reduction of the levodopa dose, thus minimizing side effects of levodopa therapy (3).

## CEREBRAL ISCHEMIA

The energy needs of the brain are supplied by metabolism of glucose and oxygen for the phosphorylation of ADP to ATP. Rapid loss of ATP occurs following cerebral ischemia, resulting in uncontrolled leakage of ions across the cell membrane, membrane depolarization and release of neurotransmitters glutamate and dopamine (64). Excess glutamate release and stimulation of its receptors results in phospholipases activation, phospholipid hydrolysis and arachidonic acid release (5), ultimately leading to apoptotic or necrotic cell death (65). Apoptotic cell death is mediated by activation of caspases. CDP-choline attenuated expression of pro-caspases, cleaved caspase-3 and nuclear DNA fragmentation after focal cerebral ischemia (27). CDP-choline in combination with nimodipine reduced infarction and increased expression of anti-apoptotic Bcl-2 after focal cerebral ischemia (26). CDP-choline pre-treatment prevented excitotoxic death caused by excessive glutamate exposure in cerebellar granule neurons (45) and in *in vivo* focal cerebral ischemia model (101). These findings have been summarized in Fig. 1A.

### Phospholipases

In addition to being an essential membrane structural component, PC is the source of bioactive lipids such as phosphatidates, 1,2-diacylglycerol, and arachidonic acid, among others (58). Thus, PC hydrolysis serves important roles in signal transduction mediated by various stimuli

including cytokines (59,84). PC can be hydrolyzed (66,67) by PC-phospholipase C (PLC) (68), PC-phospholipase D (PLD) (69), or $PLA_2$ (70). There is substantial evidence that $PLA_2$ is activated in transient ischemia and contributes to neuronal damage (12,24).

$PLA_2$ isozymes occur in multiple forms (71,72) in the mammalian cell and are classified as calcium independent, cytosolic ($cPLA_2$) and secretory ($sPLA_2$). $sPLA_2$ isoforms are low molecular weight (~14 kDa) and require millimolar $Ca^{2+}$ concentrations for activity. Our studies demonstrated significant increases in $PLA_2$ activity in membrane and mitochondrial fractions following transient cerebral ischemia (13,24). The majority of $PLA_2$ activity required mM $Ca^{2+}$ for optimal activity, characteristic of $sPLA_2$. CDP-choline treatment significantly attenuated $PLA_2$ activity in both membrane and mitochondrial fractions. *In vitro*, CDP-choline and its components cytidine and choline had no effect on $PLA_2$ activity, and thus CDP-choline is not as such a $PLA_2$ inhibitor (13). Since CDP-choline does not directly inhibit $PLA_2$, our data suggests that CDP-choline prevents activation of $sPLA_2$ (Fig. 1B). These findings are consistent with our data that CDP-choline attenuated loss of phospholipids and increase in free fatty acids including arachidonic acid after both focal and global transient cerebral ischemia (14–16,24).

### TNF-α and IL-1β Disrupt PC Homeostasis

Brain PC homeostasis is regulated by a balance between synthesis and hydrolysis by phospholipases. Two forms of IL-1 are present in brain tissue, IL-1α and IL-1β, and act on the IL-1 receptor (73). TNF-α and IL-1β are induced following brain ischemia (74–77). TNF-α (78–81) and IL-1 (82–88) stimulate hydrolysis of PC through induction of $PLA_2$ and PC-phospholipase C (PC-PLC), which mediate their cytotoxicity (84,86,87). Activation of $PLA_2$ by TNF-α/IL-1 may be mediated by increases in $PLA_2$ activating protein (PLAP) (88–90). Treatment with TNF-α antibody attenuated infarction (24,77), loss of phospholipids and increase in ceramide and free fatty acids including arachidonic acid after focal cerebral ischemia (24).

### CCT and Lyso-PC (4)

With the exception of liver, the CDP-choline pathway is the main source of *de novo* PC synthesis in mammalian tissues. Thus, CCT is a key enzyme in regulation of PC synthesis in non-hepatic tissues including the brain. CCT is an amphitropic enzyme in mammalian tissue and is distributed between cytosol and membrane. The catalytic activity of CCT has an absolute requirement for a lipid environment, which is provided by the phospholipids of the cellular membrane. Lyso-PC, a $PLA_2$ hydrolysis product of PC, inhibits CCT (Fig. 2A) (91).

TNF-α can also stimulate proteolytic degradation of CCT, resulting in a decrease in CCT protein (Fig. 2A) (93). Thus, TNF-α/IL-1 disrupt PC homeostasis by increasing PC hydrolysis (increase $PLA_2$ and PC-PLC activities) and inhibiting its synthesis (decrease CCT activity) (Fig. 2B).

We have shown stimulation of $PLA_2$, loss of phospholipids including PC (13–15,24), decrease in CCT activity and increase in lyso-PC (4) following transient brain ischemia. CDP-choline significantly increased CCT activity (4) and attenuated lyso-PC. Thus, many of the effects of CDP-choline are in a direction opposite to those of TNF-α/IL-1; one hypothesis is that the beneficial effects of CDP-choline result by partly counteracting TNF-α/IL-1 mediated events (Fig. 2B). However, further studies are required to validate this hypothesis.

These findings may have important implications for treatment of ischemic brain injury with CDP-choline. Loss of CCT was an early event in cerebral ischemia that persisted throughout 1 day reperfusion. While CDP-choline increased CCT activity in the membrane fraction, this

did not occur until 6-h reperfusion. Thus, even with CDP-choline treatment, PC synthesis may be impaired for several hours after cerebral ischemia.

CDP-choline treatment delayed by 3 h did not offer any neuroprotection (8). This later treatment could further delay the recovery of CCT activity during the critical early reperfusion time. Identification of the factor(s) responsible for CCT loss may lead to new therapeutic interventions in stroke, or combination treatments that could enhance CDP-choline's efficacy. Cholinephosphotransferase (CDP-choline-1,2-diacylglycerol cholinephospho-transferase (CPT), EC 2.7.8.2) catalyzes the final step of the Kennedy (CDP-choline) pathway for the *de novo* synthesis of PC. Though CPT is non-rate limiting under normal conditions, it may act as regulatory switch under some pathological conditions and set in motion the apoptotic cell death program (102,103). The role of this enzyme in CNS injury and the effect of CDP-choline need to be examined. Emerging lipidomics (104,105) may offer some solutions on elucidating CDP-choline mechanisms through integration of cytokine biology and lipid metabolism (106,107) in the near future.

### Reactive Oxygen Species (ROS) Lipid Peroxidation and Glutathione

Formation of ROS and the resultant oxidation of biological molecules is a well-recognized mechanism of tissue damage in ischemia/reperfusion (65,94). ROS induce lipid peroxidation, resulting in formation of malondialdehyde (MDA), 4-hydroxy-nonenal (HNE) and acrolein (95). HNE and acrolein induce neuronal apoptosis by covalently cross-linking with proteins (95,96). CDP-choline significantly attenuated ischemia-induced hydroxyl radicals ($OH^{\bullet}$) and MDA formation (12,13). $PLA_2$ releases free fatty acids including arachidonic acid from membrane phospholipids. Arachidonic acid metabolism by COX/LOX is considered to be a significant source of ROS (12,17,97). The other important free fatty acid, docosahexaenoic acid (DHA) is a major source of ROS, lipid peroxidation and neuronal injury. Bazan's group (106,107) showed some elegant relationship between pro-inflammatory gene expression and lipid metabolism by tandem liquid chromatography-photodiode array-electrospray ionization-mass spectrometric-mass spectrometry (LC-PDA-ESI-MS-MS) using lipidomic (104,105) approaches. The decrease in $OH^{\bullet}$ production in transient brain ischemia following CDP-choline treatment may be due to attenuation of $PLA_2$ activation. Glutathione is the primary endogenous antioxidant defense system and removes $H_2O_2$ and lipid peroxides in the brain. CDP-choline increased total glutathione levels, glutathione reductase activity, decreased GSSG and glutathione oxidation ratio (an indicator of the redox status of glutathione) after transient cerebral ischemia (15).

## SINGLE DRUG INTERVENTIONS MAY NOT BE EFFECTIVE

Due to the multiple pathways involved in ischemic injury, no single agent is likely to provide complete neuroprotection (98,99). CDP-choline in combination with NMDA receptor antagonist MK801 (100), thrombolytic agent (recombinant tPA) (29), urokinase (28), or basic fibroblast growth factor (23) showed synergistic benefit in experimental ischemia models. There seems to be a growing consensus to adopt a multi-pronged approach using drug cocktails since the nature of stroke injury is complex and multi-dimensional (98,99,108). CDP-choline is non-xenobiotic, safe and well tolerated, which makes it a viable choice to be used in combinational therapy for the treatment of stroke and Parkinson's disease (with levodopa).

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## Abbreviations

**CDP-choline (Citicoline)**
cytidine-5′-diphosphocholine

**CMP**
cytidine 5′-monophosphate

**CCT, CTP**
phosphocholine cytidylyltransferase

**CTP**
cytidine triphosphate

**GSH**
glutathione

**IL-1**
interleukin 1

**PC**
phosphatidylcholine

**PLA$_2$**
phospholipase A$_2$

**PLC**
phospholipase C

**ROS**
reactive oxygen species

**PLAP**
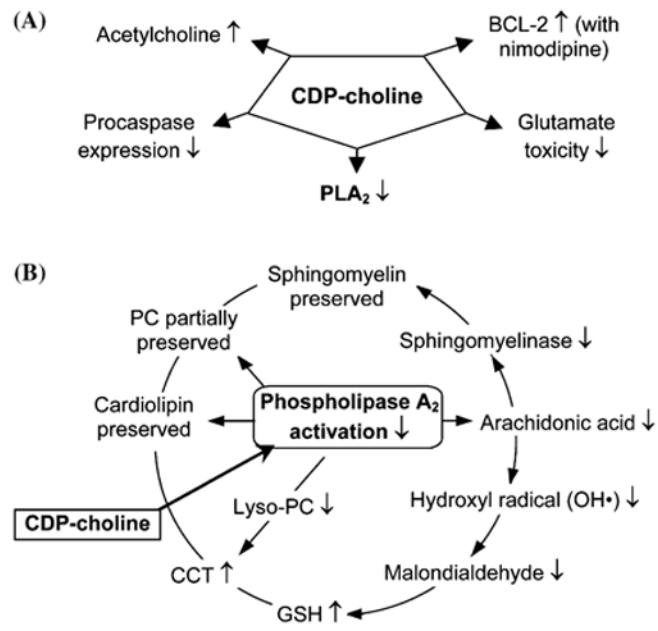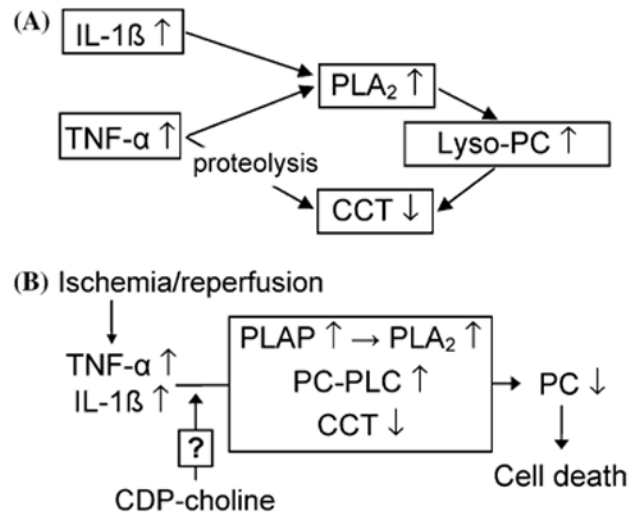PLA$_2$ activating protein

**TNF-α**
tumor necrosis factor-α

**Fig 1.**
CDP-choline: (A) possible neuroprotective pathways based on the published reports, (B) effects mediated by attenuating PLA$_2$ stimulation (based on authors' work). ↑ indicates increase; ↓ indicates decrease.

**Fig 2.**
TNF-α, IL-1β, CDP-choline and PC homeostasis. (A) Potential pathways of CCT inhibition mediated by TNF-α. TNF-α and IL-1β are induced after brain ischemia (74,77,92) and stimulate PLA$_2$ (78) and releases lyso-PC. TNF-α can inhibit CCT activity by two pathways: (1) proteolysis of CCT (93), and/ or (2) inhibition by lyso-PC (91). (B) TNF-α and IL-1β may be responsible for the loss of PC through modulation of PLA2 (78), PC-PLC (80) and CCT (84, 93) following transient cerebral ischemia. Hypothesis: CDP-choline may counteract the TNF-α/IL-1β mediated disruption of PC homeostasis by attenuating PLA$_2$ activation and increasing CCT activity (Fig. 1B). ↑ indicates increase; ↓ indicates decrease. PLAP, PLA$_2$ activating protein.

**Table I**

CDP-Choline Studies in *in vivo* (Normal and Pathological) and *in vitro* Conditions

| | Effect/Outcome by CDP-choline | References |
|---|---|---|
| *Reviews* | | |
| Pharmacology and clinical use | Review of pharmacology and clinical use | (3,6) |
| | Development for stroke trials by drug companies (Ferrer, Takeda, and Indevus, formerly Interneuron) | (7) |
| CNS injury | Mechanisms in cerebral ischemia & clinical efficacy | (5,8) |
| | Action in CNS injury and stroke trials | (9,10) |
| Glaucoma | Application for glaucoma treatment | (11) |
| *In vivo studies* | | |
| Transient forebrain ischemia in gerbil | Affected CCT activity, lyso-PC, OH• radical, lipid peroxidation, lipids, phospholipases $A_2$ and C, arachidonic acid release, leukotriene $C_4$, GSH, and hippocampal neuronal death | (4,12–18) |
| | CDP-choline but not cytidine alone provided hippocampal neuroprotection | (19) |
| Permanent forebrain ischemia in gerbil | Intracerebroventricular pre-treatment affected brain lipids | (20) |
| Transient global ischemia in rat | Liposome encapsulation decreased lipid peroxidation and increased survival | (21) |
| | Restored glucose metabolism and increased acetylcholine synthesis | (22) |
| Transient focal cerebral ischemia in rat | Combination with MK-801 or basic fibroblast growth factor synergistically reduced infarction | (23) and references therein |
| | Attenuated $PLA_2$ activity, phospholipid loss and increase in FFA and ceramide | (24) |
| Decapitation ischemia in rat | Effects on lipids, $Na^+$–$K^+$-ATPase and acetylcholinesterase activities | (25) |
| Permanent focal cerebral ischemia in SD rat | In combination with nimodipine reduced apoptosis, infarct size, and increased Bcl-2 expression | (26) |
| | Attenuated caspase cleaved products of PARP and DNA fragmentation | (27) |
| Embolic ischemic stroke in rat | Combination with urokinase improved neurobehavioral score and reduced infarction | (28) |
| | Combination with rtPA promoted functional recovery and reduced infarction | (29) |
| Subarachnoid hemorrhage | Neuroprotection could be mediated by recovery of arterial pressure in hypotensive rats | (30) |
| Cardiovascular effects on rat | Increased blood pressure and reversed hypotension in hemorrhagic shock | (31) |
| Traumatic brain injury in rat | Attenuated blood-brain barrier dysfunction, edema, cortical contusion volume, $CA_3$ neuronal death, and neurological dysfunction | (32,33) |
| | Improved cognitive deficits | (34) |
| Parkinson's disease model in rat | Attenuated substantia nigra dopaminergic cell dropout and tyrosine hydroxylase immunoreactivity | (35) |
| β-amyloid deposit + hypoperfusion in rat | Attenuated hippocampal neuronal apoptosis, and reduced microglia activation | (36) |
| Rat | Reduced benzodiazepine-induced cognitive impairments | (37) |
| | Increased CCT activity and decreased platelet activating factor | (38) |
| Female rats | Protected selective impairment in hippocampal deficits | (39) |
| | PC levels increased only after 42 days of CDP-choline administration | (40) |
| Intracerebral hemorrhage in Swiss albino mice | Improved functional outcome, reduced infarct volume | (41) |
| Hypoxia in guinea pig | Affected phospholipid biosynthesis in brain mitochondria. | (42) |
| Dog | Increased memory and learning | (43) |
| *In vitro studies* | | |
| PC12 cells | Cytidine + choline stimulated phospholipid production, expression and secretion of amyloid precursor protein | (44) |
| Cerebellar granule neurons/MCAO | Prevented glutamate mediated cell death | (45,101) |
| Human neuroblastoma SH-SY5Y cells | Increased glutathione redox ratio and attenuated caspase-3 activation in staurosporine-treated cells | (46) |
| Mouse retinal ganglion cells | Showed protective effects on damaged retinal ganglion. | (47) |
| Rat brain homogenates | Stimulated acetylcholinesterase and $Na^+$–$K^+$-ATPase activities in tissue homogenates | (48) |
| *Clinical studies* | | |
| Stroke | Review of US clinical trials. Oral treatment of patients within the first 24 h after stroke increased probability of recovery at 3 months | (49 and references cited therein) |
| Alzheimer's disease | Improved mental performance in Alzheimer's disease | (50) |
| Parkinson's disease | In combination with levodopa showed functional improvement and decreased side effects of levodopa | (3) |
| Normal subjects | Increased choline and uridine but not cytidine levels | (51) |
| Memory | Improved memory deficits in elderly subjects | (52) |
| Amblyopia/glaucoma | Improved the visual activity, function in patients | (11) |
| Drug addiction | Reduced craving for cocaine | (53) |
| Alcoholism | Improved concentration in patients with alcoholic abstinence syndrome | (3) |