

Neuroprotective Effect of Citicholine in Mercury Intoxication

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Submission date: 29-Aug-2020 12:45PM (UTC+0800)

Submission ID: 1375866963

File name: europrotective_Effect_of_Citicholine_in_Mercury_Intoxication.pdf (480.96K)

Word count: 4479

Character count: 23749

Neuroprotective Effect Of Citicholine In Mercury Intoxication

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ABSTRACT

Objective: Mercury induces cell death in various cell lines by apoptosis or necrosis. It is associated with alteration of some apoptosis regulators such as p53 and caspase-3. Citicholin has been widely used as neuroprotectant in cerebrovascular disease and other neurologic disorder. Previous studies have shown beneficial effect of citicholin in reducing inflammation and apoptosis in patient with stroke and pain, but none of those have used mercury intoxicated rat animal model. We try to investigate the neuroprotective effect of citicholin mercury intoxication.

Methods : We conducted an controlled study using male rats (*Rattus Novergicus*) as animal model and divided the subjects into 5 groups (P0,P1,P2,P3,P4) and 0.4 mg/kg BW of MeHgCl3 is administered for 21 days to all groups. Citicholin (0,5,10,20 and 40 mg/kgBW/day was added during the exposure. Protein p53 and Caspase-3 expression on neurons were counted .

Result : The mean scores±SD of p53 in group P0;P1;P2;P3;P4 were 8,406±0,70; 6,828±1,40; 4,680±1,2; 1,752±0,4; 0,807±0,1 respectively and for Caspase 3 were 8,854±1,91; 6,670±0,61; 4,21±1,02; 2,632±1,00; 0,818±0,22 respectively.

Conclusion : citicoline treatment exerts neuroprotective effect against neuronal apoptotic changes by reducing the expression of p53 and caspase-3 in dose dependent manner on mercury intoxicated rat.

KEYWORD : mercury, citicoline, p53, caspase-3

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I. INTRODUCTION

Apoptosis or programmed cell death is a normal component in the physical development and health physiologic process in multicellular organism starting in the early life until elderly[1]. This remarkable process is responsible for cell death in development, normal tissue turnover, and also accounts for many cell deaths following exposure to cytotoxic compounds, hypoxia or viral infection, death cell in tumor, injury, and other hazardous substances[2, 3]. Apoptosis is highly programmed cell death and regulated by many regulators and processes whereas the cell itself is actively participate in the process. When apoptosis become uncontrolled, it will devastate the balance and end up in a variety of malignant disorder such as myocard ischemic, neurodegenerative disease, stroke, shock septic[4]. Regulation of apoptosis is very complicated involving apoptotic and antiapoptotic factors. Apoptosis can be triggered via stimulation of several different cell surface receptors in association with caspase activation through either extrinsic or intrinsic pathway. Normally the death cell program will occur during embriogenesis and acute inflammation.

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There is growing evidence that mercury exert neurotoxic effect on human brain, mercury induces cell death in various cell lines and primary cell culture by apoptosis or necrosis or a mixture of the two in a time and dose-dependent manner. Both inorganic and organic mercury are potent human cell apoptogens. Mercury seems to be associated in alteration of some regulators of apoptosis. Some researchers have proposed that mercury could alter the level of pro-apoptotic protein p53 and caspase 3[5]. p53 is a transcription factor which function as regulator of cell cycle progression and apoptotic process[6, 7]. This factor is upregulated in response to various cellular stresses and can direct cell to undergo apoptosis[8, 9]. Alteration in p53 expression has been associated with neuronal damage in a variety of in vivo model system[10]. p53 is activated by a variety of stressful cellular conditions, including DNA damage, oxidative stress, and oncogenic signals[11].

Caspases are (CysteinyI-aspartate-specific proteinases) and divided into two general initiator and effector group, caspase 3 is the effector protein which require cleavage by initiator caspases for activation. Once activated caspase-3 will initiate cell degradation process. Cytidine-5-diphosphocholine (CDP-choline or citicholine) is an essential intermediate in the synthesis of phosphatidylcholine, a major brain phospholipid [12]. CDP-choline has been shown to reduce cell-membran breakdown and free radical generation. Previous studies have shown beneficial effect of CDP-choline in reducing inflammation and apoptosis. CDP-choline lowers expression of procaspase -1,-2,-3,-6,-8 and caspase-3 hence reduces apoptosis. Other mechanism is involving NF-kB pathway, CDP-cholin treatment decreases sPLA₂(secretory Phospholipase A₂), mRNA trancription and inhibits sPLA₂ enzym. Activation of sPLA₂, mRNA will lead to PtdCho hydrolysis result in membran damage, increasing ROS and ceramid production. Ceramid is known to trigger apoptosis [13]. The neuroprotective effect citicolin has been widely studied in stroke and proved to be beneficial. The researchers used various method and parameter in inducing apoptosis and measure the outcome. Krupinski et al used in vitro angiogenesis assays in human brain microvessel endothelial cells (hCMEC/S3) and induced transient MCAO in rat brain. Citicholin treatment were added and parameters such as expression of phospho-extraellulaar-signal regulated kinase (ERK)-1/2, insulin receptor substrated 1(IRS-1) which showed proangiogenic effect were counted whereas apoptotic cell were measured to reveal neuroprotective effect [Krupinski, 2012]. Sahin S et al used Middle cerebral artery occlusion in rats and measured Bcl2 and caspase 3, Bax, caspase 9 after treatment with citicohlin to show the supreession effect of citicholin on apoptotic precesses [Sahin et al, 2010]. Sabrado et al used combined nimodipine and citicoline treatment in ischemia induced and scored the infarcted tissue, Bcl-2 expression to determine the level of apoptosis [14]. Alvaraz et al uses rats subjected to implants of the beta-amyloid fragment 1-40 (A beta 4:3 Mmol) in the right hippocampus and cerebral hipoperfusion to induced neuro degeneration and apoptosis and measure the number of apoptotic figures (tunel technique) were evaluated to determine the effect of citicoline. All researches as far as we are aware, no-one has used mercury intoxicated rat model to reveal beneficial effect of citicholin in reducing apoptosis. We conducted trial of true experimental study with the post test only control group design investigating the role of CDP-cholin in protecting neuron from apoptosis process of mercury intoxicated rat. The implication of this study would suggest the potential clinical application of CDP-cholin in mercury intoxication.

II. MATERIAL AND METHODS

Setting : This experiment has been conducted in animal research laboratory of school veterinary, Airlangga University, Surabaya and analysis has been carried out at laboratory of school veterinary Brawijaya University, Malang, East Java, Indonesia.

Animal model : Male rats (*Rattus Novergicus*) aged 4 months, weighed 100 to 200 g were used as animal models and were exposed to methylmercury chloride via nasogastric tube. Subjects were divided into 5 groups P0 (control); P1; P2; P3; P4. MeHgCl₃ of 0.4 mg/kg BW is administered for 21 days to all group. Citicoline with the dose of 5 mg; 10 mg; 20 mg; 40 mg/kgBW/day was added to P1; P2; P3; P4 group during the exposure. After the treatment, the brains (hemisphere cerebri) of the subjects were removed, sliced and processed to measure p53 and Caspase-3 count. Methylmercurychloride and Citicoline were given at the same time, each in 0,5 cc dilution via nasogastric tube.

Chemical : Methylmercury(II) chloride powder, produce of Arema. Sigma-Aldrich, Co, St. Louis, USA diluted into aquabidest to get the dose of 0.4 mg/kg BW in 0,5 cc. Immunohistochemistry On day 22 brain of each model was removed, fixed in buffer formalin 10% and put on object glass, stained and ready for analyzing. Review of the specimens were done by two examiner were assign to measure the expression p53 and Caspase-3 using Axiovision rel. 4.8. The number of cells expressing p53 and caspase-3 will be measured. Equipped by Japan Olympus lens 400 x magnification was used and 10 visual fields were randomly picked up for scoring neuron. Expression of p53 in neuron after *Antibody primer polyclonal rat anti p53 immunohistochemistry staining* is demonstrated by brown stain in cytoplasm whereas caspase-3 expression can be identified by brown stain in cytoplasm after *antibody primer polyclonal rat anti Caspase 3 staining*. p53 essay Antibody primer polyclonal rat anti p53 immunohistochemistry stain is used to demonstrate p53 expression. p53 is done after sample is stained using *Standard Immunohistochemistry Staining Method, Avidin Biotin Complex (ABC) Method*. Neurons which expressed p53 were calculated using Axiovision rel. 4.8 in 10 visual fields and measure the mean score. Protocol using ABC method is detailed below.

- Clearing and rehidrate the specimen gradually and rinse in destilate water (DW) for 3x5 minutes, dry up the liquid adjusten to specimen
- Incubate slides in 3% H₂O₂ in DW for 15 min in room temperature, rinse in DW for 3x5 min and rinse again in Phosphate-buffered saline (PBS) for 3x5 min

- Incubate slides in 10% skim milk solution or 10 % normal goat for 30 min in room temperature and then rinse in PBS fro 3x5 min
- Incubate slides in antibodi anti p53, rinse in PBS solution for 3x5 min
- Incubate slides in AB II for 30 min in room temperature, at the same time incubate 10 microliter avidin and 10 microliter biotin in 1 cc PBS and then use the sections in the PBS for 3x5 min
- Incubate slides in avidin bition mixture solution for 30 min in room temperature and rinse in PBS for 3x5 min.
- Incubate slides in Diaminobenzidine (DAB) solution and analyze by microscope.
- Counter staining with commasie blue
- Dehydrate slides and then analyze by microscope

Caspase 3 essay

Antibody primer polyclonal rat anti Caspase 3 was used to identify caspase 3 in neuron. Brown stain in cytoplasm demonstrated the presence of caspase 3. By using Japan Olympus lens 400 x magnification, 10 visual fields were picked up randomly. The number of neuron which expressed caspase were calculated using Axiovision rel. 4.8 and mean score were analyzed. Immunohistochemistry protocol is in accordance with Avidin Biotin Complex (ABC) method Statistical analysis Statistical analysis was carried out using both descriptive and statisic: analytic ie test of Homogeneity of Variances, one way anova, multiple comparison, homogenous subset. p-values less than 0.05 were considered statistically significant.

III. RESULT

TABLE 1. Caspase-3

Group	Mean caspase – 3 expression in each subject					mean	Standard deviation
	I	II	III	IV	V		
PO	6,910	6,780	9,520	10,990	10,070	8,854	1,91
P1	7,400	6,810	6,820	5,730	6,590	6,670	0,61
P2	5,320	3,200	3,120	4,380	5,040	4,212	1,02
P3	2,890	1,580	2,510	4,190	1,990	2,632	1,00
P4	0,780	1,020	1,050	0,510	0,730	0,818	0,22

- Note :
- P0 : Methylmercury chloride 0,4 mg/kg BW
 - P1 : Methylmercury chloride 0,4 mg/kg BW + citicoline 5 mg/kb BW
 - P2 : Methylmercury chloride 0,4 mg/kg BW + citicoline 10 mg/kb BW
 - P3 : Methylmercury chloride 0,4 mg/kg BW + citicoline 20 mg/kb BW
 - P4 : Methylmercury chloride 0,4 mg/kg BW + citicoline 40 mg/kb BW

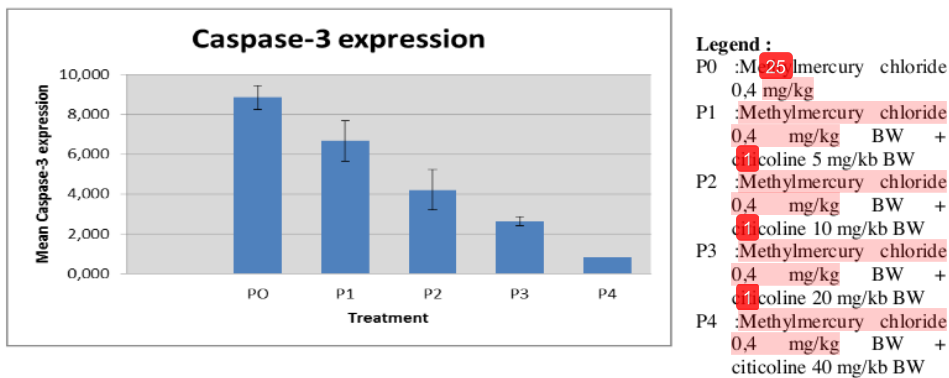


Figure 1. Result : caspase-3 expression

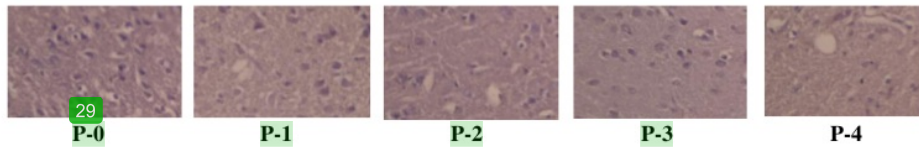


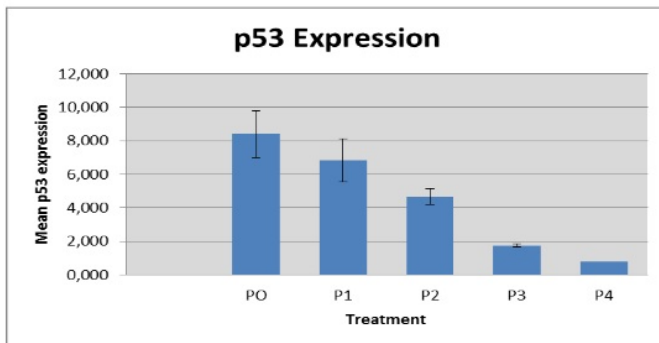
Figure 2. Image of caspase-3 expression in each group

Note : P0 : Methylmercury chloride 0,4 mg/kg BW
 P1 : Methylmercury chloride 0,4 mg/kg BW + citicoline 5 mg/kb BW
 P2 : Methylmercury chloride 0,4 mg/kg BW + citicoline 10 mg/kb BW
 P3 : Methylmercury chloride 0,4 mg/kg BW + citicoline 20 mg/kb BW
 P4 : Methylmercury chloride 0,4 mg/kg BW + citicoline 40 mg/kb BW

TABLE 2. p53 essay

Group	Mean p53 expression in each subject					Mean	Standard deviation
	I	II	III	IV	V		
PO	8,640	8,590	7,990	7,480	9,330	8,406	0,70
P1	9,070	5,620	6,310	5,870	7,270	6,828	1,40
P2	6,330	5,210	3,170	3,590	5,100	4,680	1,29
P3	2,180	2,240	1,270	1,250	1,820	1,752	0,48
P4	0,770	0,690	0,941	0,860	0,772	0,807	0,10

Note : P0 : Methylmercury chloride of 0,4 mg/kg BW
 P1 : Methylmercury chloride 0,4 mg/kg BW + citicoline 5 mg/kb BW
 P2 : Methylmercury chloride 0,4 mg/kg BW + citicoline 10 mg/kb BW
 P3 : Methylmercury chloride 0,4 mg/kg BW + citicoline 20 mg/kb BW
 P4 : Methylmercury chloride 0,4 mg/kg BW + citicoline 40 mg/kb BW



Legend :
 P0 : Methylmercury chloride 0,4 mg/kg
 P1 : Methylmercury chloride 0,4 mg/kg + citicoline 5 mg/kb BW
 P2 : Methylmercury chloride 0,4 mg/kg + citicoline 10 mg/kb BW
 P3 : Methylmercury chloride 0,4 mg/kg + citicoline 20 mg/kb BW
 P4 : Methylmercury chloride 0,4 mg/kg BW + citicoline 40 mg/kb BW

Figure 3. Result : p53 expression

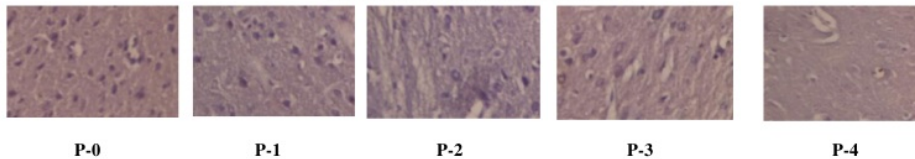


Figure 4 : Image of p53 expression in each group.

Note : P0 : Methylmercury chloride 0,4 mg/kg BW
 P1 : Methylmercury chloride 0,4 mg/kg BW + citicoline 5 mg/kb BW
 P2 : Methylmercury chloride 0,4 mg/kg BW + citicoline 10 mg/kb BW
 P3 : Methylmercury chloride 0,4 mg/kg BW + citicoline 20 mg/kb BW
 P4 : Methylmercury chloride 0,4 mg/kg BW + citicoline 40 mg/kb BW

Anova test reveals caspase 3 count between group differs significantly with p-value 0.000 30 rats have been enrolled to this study, of 30, 5(16,67 %) or 1 in each group (16,7%) died during the treatment. Table 1 demonstrates caspase-3 expression in each group. Group P0 (control) shows the highest level ($8,854 \pm 1,91$) whereas P4 group reveals the lowest ($0,818 \pm 0,22$). Group P0 was exposed to mercury with the same dose as that of group P1,P2,P3,P4 but P0 was not treated with CDP-choline as other groups were. Caspase in group P1 to P4 decrease as the dose of CDP-choline given to P1-P4 increase (from 5 to 40 mg/kgBW/day). The result demonstrates an inverse dose relationship between caspase-3 level and the dose of CDP-cholin. Test of Homogeneity of Variances demonstrates the homogeneity of caspase-3 with p-value 0.001. Anova test analyze the result between group reveals the significant different of caspase-3 level between group with p value 0.000. Similar to caspase-3 level result, table 2 demonstrated p53 has the highest level in group P0 ($8,406 \pm 0,7$) whereas its lowest level in group P4 is $0,807 \pm 0,10$. The steep down decrease from P0 to P4 is in accordance with the increasing dose of CDP-Cholin given to group P1 to P4. There is a obvious inverse dose relationship between p53 and the dose of CDP-cholin. Homogeneity using Levene statistic reveals homogeneity with p-value 0.009. Anova test analyze between group reveals the significant different of p-53 level between group with p value 0.000.

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IV. DISCUSSION

In this study we have showed the neuroprotective effect of CDP-choline on mercury intoxicated rat brain *in vivo*. Some studies has demonstratred the role of mercury inducing apoptosis in various target[15, 16] and also many of its devastating effect[17-20]. The treatment for mercury toxicity so far has been emphasized on avoiding exposure, chelation therapy as well as administration of antioxidant agent since mercury has been known to bind to sulphydryl compound thus reducing antioxidant level[19, 21-23]. None of these authors has put citicolin on a guideline to treat mercury poisoning. In cellular level mercury induces apoptosis in 3 major mechanisms : disrupting Ca^{2+} intraceluler, increasing ROS production and decreasing antioxidant level because mercury binds to sulphydryl group[24].Ethyl-mercury reduces mitochondria potential membrane, result in citochrome-c release and apoptosis inducing factor leading to apoptosis via mitochondrial dependent pathway (intrinsic pathway) which stimulate caspase cascade through activation of caspase-9 and caspase-3[25]. Shimela & Tahti also demonstrated the role of $HgCl_2$ of 0,1 μM in inducing caspase 3 after 6 hours exposure. Caspases are probably the most important effector molecules for triggering the biochemical events which lead to apoptotic cell death. Assays for determination of caspase activation can detect apoptosis earlier than many other commonly used methods. In this study we used caspase-3 to determine neurotoxic effect of mercury since caspases play a pivotal role in the initiation and execution of apoptosis induced by various stimuli. The highest caspase-3 mean level is in the P0 group ($8,854 \pm 1,91$) which is exposed to 0,4 mg MeHgCl₃ for 21 days without additional treatment. The caspase-3 level decreases gradually from P1 to P4 group along with the increasing dose of CDP-choline given to group P1 to P4. This result has revealed the strong protective effect of CDP-choline against apoptosis demonstrated by the ability to reduce the caspase-3 level in a dose-dependent manner .

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The p53 tumor suppressor acts to integrate multiple stress signals into a series of diverse proliferative responses. One of the most important p53 functions is its ability to activate apoptosis, molecule p53 stimulates a wide network of signals that act through two major apoptotic pathways. The extrinsic, death receptor pathway triggers the activation of a caspase cascade, and the intrinsic, mitochondrial and consequently caspase-mediated apoptosis.[26]. p53 induces apoptosis through its role in regulating transcription of many pro-apoptotic gen such as BAX, APAF-1, Fas-L, caspase-6, caspase-10[27]. p53 also binds to some anti-apoptotic protein which work at mitochondria. p53 can be upregulated by various stimuli such as cellular stresses DNA damage, hypoxia or aberrant oncogene expression to promote cell-cycle checkpoints, DNA repair, cellular senescence, and apoptosis[8, 9, 28].

Mercury and other metals have been known to stimulate ROS & Nitrogen species production, cell damage and also mediate activation of some sensitive transcription factor such as NF-Kb, Apaf-1 dan p53[5]. In this study p53 is used to demonstrate the neurotoxic effect of mercury since p53 level can be upregulated after exposure of mercury, and once the level rised we treated the animal models with CDP-choline to reveal its neuroprotective effect. The highest level of p53 is in group P0 and the lowest is in the group P4 where CDP-choline was given as the highest dose (40 mg/kgBW/day). The two parameters of apoptosis we use in this study ie p53 and caspase-3 gradually decrease their expression when the dose of CDP-choline is increased. Statistical analyzes shows that the expression of the two parameter differs significantly. Once again this inverse dose relationship between apoptosis parameters and CDP-cholin indicate that chronic supplementation of citicolin proved to be valuable in mercury intoxicated brain.

The result of this study is in accordance with the previous studies which have shown that CDP-cholin provides significant protection for neuron and has beneficial effects in various CNS injury models and neurodegenerative disease[29]. Although some researchers used various methods and parameters in different setting but the outcomes are always similar : CDP-cholin reduces apoptosis. Krupinski *et al* used 1000 mg /kg intraperitoneally every 3 days until 21 days in induced transient MCAO in rats and found out that the drug has increased the expression of (ERK)-1/2, IRS-1 (represents a potent modulator of α -angiogenic signalling cascades in vascular) and the percentage of survival cells[30]. Alva *et al* used male Sprague Dawley rat subjected to implants of the beta-amyloid fragmen 1-40 ((A beta 4: 3 Mmol) into the right hippocampus to induce chronic glutamate excitotoxicity which is claimed to be involved in progressive motor neuron (MN) loss in amyotrophic lateral sclerosis (ALS). They performed permanent unilateral occlusion of the carotid artery to determine effect of CDP-cholin CDP; 0, 62.5, 125 and 250 mg/kg/day intra peritoneal 2 days before and 5 days after surgery demonstrated reduction in number of apoptotic neuron (TUNEL technique)[31]. Another method to induce glutamate excitotoxicity coming from Matyja *et al* who used organotypic cultures of lumbar spinal cord obtained from 8-dayold rat pups and treated with specific glutamate uptake blocker DL-threo-beta-hydroxyaspartate (THA, Sigma) in a concentration of 100 μ M induce chronic glutamate excitotoxicity The group incubated with 100 μ M CDP-choline showed less apoptotic changes characterized by peripheral condensation and margination of nuclear chromatin[32]. Another similar result coming from a study conducted by Sahin *et al* investigating neuroprotective effect of 400 mg/kg intraperitoneal citicolin on the suppression of apoptotic processes after induced cerebral ischemic on rat brain .Apoptosis parameter such as caspase -3, Bax, caspase -9 are higher in non treated group but the difference is greater in group treated with both citicoline and hypothermia[33].

V. CONCLUSION

The result of our study and the above previous studies confirm the suggestion of neuroprotective effect of citicholin in apoptosis in different diseases. Neuroprotective effect of citicoline in apoptosis process induced by mercury has not been widely documented. Therefore future investigation is warranted to reveal its importance in mercury poisoning.

Competing Interest.

The author declare no conflicts of interest

Abbreviations

CDP Choline	: Cytidine 5-Diphosphocholine
NF-kB	: Nuclear Factor kappa B
sPLA ₂	: secretory Phospholipase A ₂
mRNA	: messenger RNA
PLA	: Phospholipase.
Ptd Cho	: Phosphatidylcholine
ROS	: Reactive Oxygen Species
ABC	: Avidin Biotin
DAB	: Diamino benzidine
APAF-1	: Apoptotic protease activating factor 1

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