ผลของเจลดานามัยซินต่อการบาดเจ็บของเซลล์ประสาทจากสภาวะโพแทสเซียมต่ำในเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาว

นางสาว อรุณี คำดี

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EFFECTS OF GELDANAMYCIN ON LOW POTASSIUM-INDUCED INJURIES IN CULTURED RAT CEREBELLAR GRANULE CELLS

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สถาบันวิทยบริการ
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เจลดานามัยซิน (GA) เป็นสารธรรมชาติกลุ่ม benzoquinone ansamycin antibiotic ที่มีฤทธิ์ต้านมะเร็ง การศึกษานี้ออกแบบเพื่อทดสอบผลป้องกันเซลล์ประสาทที่อาจเป็นไปได้ของ GA ต่อการบาดเจ็บและตายของเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาว อันเกิดจากสภาวะโพแทสเซียมต่ำ โดยใช้การเมตาบอลิสมและการอยู่รอดของเซลล์ (วัดโดย MTT reduction และ LDH release) ปริมาณของ glutathione และการเกิด lipid peroxidation เป็นตัวชี้วัด เมื่อ incubate เซลล์ประสาทเพาะเลี้ยงเป็นเวลา 12-72 ชั่วโมงกับ GA ความเข้มข้นต่างๆ (0.01-1.0 μM) พบว่าเกิดผลป้องกันเซลล์ประสาทซึ่งขึ้นกับความเข้มข้นและระยะเวลาที่สัมผัสกับ GA โดยที่ความเข้มข้นต่ำและระยะเวลาสัมผัสที่สั้น GA มีผลน้อยต่อเมตาบอลิสมของเซลล์ประสาท อย่างไรก็ตามหลังจากสัมผัสกับ GA เป็นเวลา 24-48 ชั่วโมง ที่ความเข้มข้นสูงกว่า 0.05 μM GA มีผลกระตุ้นเมตาบอลิสมของเซลล์ประสาทอย่างชัดเจน เมื่อเซลล์ประสาทสัมผัสกับ GA เป็นเวลา 24 ชั่วโมง ที่ความเข้มข้นสูงกว่า 0.025 μM จะลดการตายของเซลล์อย่างมีนัยสำคัญ ในขณะที่การอยู่รอดของเซลล์ลดลงเมื่อเซลล์ประสาทสัมผัสกับ GA ที่ความเข้มข้นสูงขึ้นและระยะเวลาที่นานขึ้น จะเห็นได้ชัดว่าผลป้องกันเซลล์ประสาทจะเกิดสูงสุดถ้าเซลล์สัมผัสกับ GA เป็นเวลา 24 ชั่วโมง ที่ความเข้มข้น 0.10 μM

หลังจากสลับเซลล์ประสาทแกรนูลเพาะเลี้ยงจาก medium ซึ่งปราศจากซีรัมและมี K^+ 25 mM (high K^+) มายัง medium ซึ่งปราศจากซีรัมแต่มี K^+ เพียง 5 mM (low K^+) เป็นเวลา 24 ชั่วโมง ปรากฏว่าประมาณ 50% ของเซลล์ประสาทตาย การสัมผัสกับ GA พร้อมกับภาวะ low K+ เป็นเวลา 24 ชั่วโมงไม่มีผลป้องกันการตายของเซลล์ประสาทดังกล่าว ในขณะที่การสัมผัสกับ GA ที่ความเข้มข้น 0.025-0.10 μM ล่วงหน้าเป็นเวลา 24 ชั่วโมงก่อนสัมผัสกับภาวะ low K^+ เป็นเวลา 24 ชั่วโมง มีผลกระตุ้นการเมตาบอลิสมของไมโตคอนเดรียและป้องกันการตายของเซลล์ประสาทอย่างมีนัยสำคัญ จากการวิเคราะห์ GSH แสดงว่าปริมาณ GSH ในเซลล์ลดลงเกือบ 40% เมื่อสลับเซลล์ประสาทเพาะเลี้ยงมาอยู่ในภาวะ low K^+ เป็นเวลา 24 ชั่วโมง โดยที่การสัมผัสกับ 0.1 μM GA ล่วงหน้าเป็นเวลา 24 ชั่วโมงสามารถป้องกันการลดลงของ GSH ที่เกิดขึ้นได้อย่างมีประสิทธิภาพ นอกจากนั้นการสัมผัสกับ GA ล่วงหน้าดังกล่าวสามารถป้องกันการเพิ่มขึ้นของ lipid peroxidation อันเกิดจากภาวะ low K^+ ได้ โดยสรุปข้อมูลเหล่านี้ชี้แนะว่าการสัมผัสกับ GA ล่วงหน้าอาจป้องกันการตายของเซลล์ประสาทแกรนูลจากภาวะ low K^+ ได้ด้วยการเหนี่ยวนำการสังเคราะห์โปรตีนที่กำกับควบคุมการเมตาบอลิสมของ GSH และขบวนการ lipid peroxidation

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4376611433 MAJOR: PHARMACOLOGY
KEYWORDS: GELDANAMYCIN/CEREBELLAR GRANULE CELLS/LOW POTASSIUM/APOPTOSIS

ARUNEE KUMDEE: EFFECTS OF GELDANAMYCIN ON LOW POTASSIUM-INDUCED INJURIES IN CULTURED RAT CEREBELLAR GRANULE CELLS. THESIS ADVISOR: ASST. PROF. SURACHAI UNCHERN, Ph.D., 104 pp. ISBN 974-17-5366-7

Geldanamycin (GA) is a natural benzoquinone ansamycin class antibiotic which possesses antitumor activity. This study was designed to investigate a suspected and potential neuroprotective effect of GA against K^+ deprivation-induced cell injury and death in primary cultured rat cerebellar granule cells. Cell metabolic activity and viability (assessed by MTT reduction and LDH release), content of glutathione and lipid peroxidation, were used as the measuring endpoints. Exposure of cultured cerebellar granule neurons for 12-72 hr to geldanamycin at different concentrations (0.01-1.0 μM) showed concentration- and exposure time-dependent neuroprotective effects. At low concentrations of GA with short incubation periods, GA showed a little effect on neuronal metabolic activity. After 24-48 hr pre-exposure to GA at concentrations higher than 0.05 μM, GA had a marked effect on neuronal metabolic activity whereas an exposure to very high concentrations and prolonged duration caused reduction in cell survival. With 24 hr pre-exposure to GA at concentrations higher than 0.025 μM, cell death was significantly prevented. Apparently, the maximal magnitude of neuroprotection may be obtained from 24 hr pre-exposure to 0.10 μM GA.

After switching cultured cerebellar granule neurons from a serum medium containing 25 mM K^+ (high K^+) to a serum-free medium containing 5 mM low K^+ (low K^+) for 24 hr, approximately 50% of neurons had loss their viability. Simultaneous exposure with GA and low K^+ for 24 hr did not protect neuronal cell death while pretreatment with 0.025-0.10 μM GA for 24 hr before shifting to low K^+ medium significantly boosted up mitochondrial metabolic activity and prevented neuronal cell death in low K^+ condition. Furthermore, GSH assay revealed that total GSH content was reduced by nearly 40% after switching cultured neurons to low K^+ for 24 hr. Pretreatment with 0.1 μM GA for 24 hr effectively inhibited low K^+-induced GSH diminution. Moreover, the same pretreatment with GA also prevented an increase in cellular lipid peroxidation after 24-hr exposure to low K^+. These findings suggest that GA pretreatment may prevent granule cell death by induction of proteins that regulate GSH metabolism and lipid peroxidation.

Department of Pharmacology Student's signature.....

Field of study Pharmacology Advisor's signature.....

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LIST OF ABBREVIATIONS

β	=	beta
°C	=	degree celcius
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
AA	=	arachidonic acid
AMPA	=	α-amino-3-hydroxy-5-methyl-ioxyzole-4-propionic acid
$BaCl_2$	=	barium chloride
BDL	=	bile duct ligation
BDNF	=	brain-derived neurotrophic factor
Ca^{2+}	=	calcium ion
CCl_4	=	carbon tetrachloride
CGNs	=	cerebellar granule neurons
CNS	=	central nervous system
CNTF	=	ciliary neurotrophic factor
DMEM	=	Dulbecco's Modified Eagle's Medium
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
DPBS	=	Dulbecco's phosphate buffered saline
DPPH	=	1,1-diphenyl-2-picrylhydrazyl
DTNB	=	5,5'-dithiobis (2-nitrobenzoic acid)
ER	=	endoplasmic reticulum
et al.	=	et alii (and other)
FBS	=	fetal bovine serum
FGFs	=	fibroblast growth factors
GA	=	geldanamycin
GSH	=	glutathione
GSSG	=	glutathione disulfide
HIV	=	human immunodeficiency virus
HSF	=	heat shock factor

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HSPs	= heat shock proteins
IC_{50}	= median inhibition concentration
IGFs	= insulin-like growth factors
K^+	= potassium ion
KCl	= potassium chloride
kg	= kilogram
L	= litre
LDH	= lactate dehydrogenase
MDA	= malondialdehyde
mg	= milligram
mM	= millimolar
MTT	= 3-(4,5 – dimethylthiazol – 2- yl) – 2,5 – diphenyltetrazolium bromide
Na^+	= sodium ion
NADP	= nicotinamide adenine dinucleotide phosphate
NADPH	= nicotinamide adenine dinucleotide phosphate (reduced form)
NGF	= nerve growth factor
nm	= nanometer
NMDA	= N-methyl-D-aspartate
nmol	= nanomole
NOS	= nitric oxide synthase
NT3, NT4	= neurotrophins 3 and 4
PBS	= phosphate buffered saline
RNA	= ribonucleic acid
ROS	= reactive oxygen speices
s-ALP	= serum levels of alkaline phosphatase
s-ALT	= serum levels of alanine aminotransferase
s-AST	= serum levels of aspartate aminotransferase
SEM	= standard error of mean
SOD	= superoxide dismutase
TBA	= thiobarbituric acid
TBARS	= thiobarbituric acid reactive substance
TLC	= thin layer chromatography
TNB	= 5-thio-2-nitrobenzoate

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CHAPTER I

INTRODUCTION

Central nervous system (CNS) is highly susceptible to damage by a variety of biological agents. This problem is enhanced by the fact that neurons and neuron-derived cells, with few exceptions, do not renew themselves so a gradual reduction in these essential elements throughout a lifetime is unavoidable. The insidious reduction in the number of neurons and their synaptic connections eventually compromises virtually all CNS functions. Because of this, a prudent approach would be to minimize neuronal loss and thus forestall the associated neurophysiological and neurobehavioral decrements that are seemingly inevitable in the aged. This would be of particular importance under the current circumstances where improvements in medical sciences have ensured progressively greater longevity in succeeding generations of humans thereby rapidly increasing the number of individuals reaching advanced age in many well developed countries of the world.

In addition to physiological aging, humans are unfortunately subjected to neurotoxicity during their lifetime. Neurotoxicity can be defined as the capacity of chemical, biological, or physical agents to cause adverse functional or structural changes in the nervous system. Chemical exposure may adversely affect the structure of the nervous system leading to a neuronopathy, an axonopathy, or may cause functional alterations that can be detected as neurobehavioral, neurochemical, or neurophysiological changes. These changes are major deficits frequently observed in patients with neurodegenerative diseases, e.g., Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, and spinal muscular atrophy.

The death of central neurons has been widely recognized as a normal feature of vertebrate development. Programmed cell death (PCD) has a key role in normal development and throughout the entire life of any multicellular organisms. During embryogenesis, neuronal cell death adjusts the number of neurons to the size of the target innervated. When neurons fail to receive adequate trophic support they die, thus sculpting the neuronal population to match the requirement of the target. Previous studies suggest that some forms of developmental PCD in the mammalian brain occur via apoptosis, a physiological mechanism by which a cell dies via transcriptional and

translational activation of an intrinsic cell death or suicide program (Raff et al., 1993). In addition to the role of PCD in the differentiation and organization of the nervous system during its development, PCD is likely involved in the pathogenesis of degenerative neurological disorders, such as Alzheimer's disease (Cotman and Anderson, 1995), Huntington's disease (Portera-Cailliau et al., 1995), amyotrophic lateral sclerosis (Rabizadeh et al., 1995), and spinal muscular atrophy (Roy et al., 1995), in which neuronal loss is a prominent feature.

Oxidative stress is considered to be an important causative factor in the onset or progression of neurodegenerative diseases and may contribute to neuronal damage resulting from cerebral ischemia (Coyle and Puttfarcken, 1993). Reactive oxygen species (ROS), which are generated as byproducts of many metabolic processes, including monoamine metabolism (Maher and Davis, 1996), the mitochondrial electron transport chain (Tan et al., 1998a) and arachidonic acid oxidation (Li et al., 1997b), may be the principal mediators of cell death in oxidatively stressed neuronal cells (Chan, 1996). The damage imparted on various macromolecules by chemical reactions with ROS can initiate an apoptotic program of cell death (Liu et al., 1996) or lead to cell death by necrosis (Choi, 1996).

In vitro models that recapitulate neuronal apoptosis are essential for studying the molecular mechanisms underlying cell death, and may prove useful for developing neuroprotective agents as well as therapeutic interventions for neurodegenerative disorders. One of the best in vitro models of neuronal apoptosis is represented by the change of culture conditions of cerebellar granule neurons (CGNs). This neuronal population, cultured in 25 mM KCl-containing medium, develops typical in vitro features of mature CGNs in vivo, such as an extensive neuritic network, expression of excitatory amino acid receptors, and production and release of L-glutamate (Burgoyne et al., 1993). When deprived of depolarizing levels (25 mM) of extracellular K^+, CGNs undergo apoptosis (Gallo et al., 1987), showing typical cell shrinkage, chromatin condensation, pyknosis, and nucleosomal size DNA fragmentation (D'Mello et al., 1993, 1998; Galli et al 1995; Schulz et al., 1998). These are the conformational and physiological hallmarks of granule cell "programmed death" in which an alteration of reactive oxygen species (ROS) metabolism and cellular redox state has been implicated as mediator of CGN apoptotic death (Hockenbery et al.,

1993; Simonian and Coyle, 1996; Atlante et al., 1998). In this connection, the model of apoptotic neuronal death which consists of shifting K^+ concentration in the medium of cultured rat cerebellar granule cells from 25 to 5 mM has been widely used in neurotoxicological studies.

Geldanamycin (GA), a benzoquinone ansamycin antibiotic produced by *Streptomyces hygroscopocus* var. *geldanus*, is strongly cytotoxic against tumor cells, fungi and protozoa, as well as having weak anti-microbial activity (DeBoer et al., 1970). Recent study showed that very low concentrations of geldanamycin (0.5-2.0 nM) enhanced the survival and neurite outgrowth of cultured dorsal root ganglion (DRG) neurons (Sano, 2001). Cytostatic drugs, such as cisplatin, vincristibe, and taxol are widely used in the treatment of various neoplasms. When these drugs are given in high doses to cancer patients, they cause irriversible toxic neuropathies in peripheral neurons. Therefore, there has been a great interest in therapeutic agents that can reduce these side effects. Consequently, these chemotherapeutic agents have been widely studied as toxic models of neuropathy. Addition of cytostatic drugs to the cultured embryonic DRG neurons decreased the outgrowth of neurites and induced apoptosis of the neurons. Geldanamycin was found to rescue cultured sensory neurons from apoptotic cell death by such antitumor drugs with sufficient trophic support by neurotrophins (Sano, 2001). This result suggests that geldanamycin has a therapeutic potential for neurodegenerative diseases, especially for anti-cancer drug-induced sensory neuropathy.

Previous study also showed that geldanamycin is effective in preventing glutamate-induced oxidative toxicity in the HT22 mouse hippocampal cell line, even if given 4 hr after glutamate treatment. Thus, geldanamycin may provide an effective strategy for manipulating signaling pathways in neuronal cells that use HSP90 as they proceed through a programmed cell death pathway in response to oxidative stress (Xiao et al., 1999).

From these interesting activities of geldanamycin, it is reasonable to investigate this natural product in term of its potential neuroprotective property in a well-characterized model of neurodegeneration. Therefore, the present study was carried out to determine effects of geldanamycin on low potassium-induced injuries in primary cultured rat cerebellar granule neurons. In this study, effects of geldanamycin

were compared with trolox, a potent antioxidant compound, by using biochemical analyses including MTT reduction assay, lactate dehydrogenase (LDH) release assay, thiobarbituric acid reactive substances (TBARS) assay and total glutathione assay.

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CHAPTER II

LITERATURE REVIEW

Cerebellum

The cerebellum (latin, little brain) is one of the most interesting structures in the central nervous system. First, by weight it constitutes only 10% of the total brain, yet it contains more than half of all neurons in the brain. Second, it has a highly regular structure. The cerebellar cortex has an almost crystalline organization suggests that all areas of the cerebellum perform a function on a different set of inputs. Third, the cerebellum is not necessary for basic perception or for the movement of muscle. Although both the sensory systems and the motor systems are mapped onto the cerebellum, complete destruction of the cerebellum produces no sensory impairment, and muscle strength typically remains intact. Rather, the cerebellum disrupts coordination of limb and eye movements, impairs balance, and decreases muscle tone. The signs of cerebellar damage thus differ dramatically from those of damage to the motor cortex (upper motor neuron disease, which reduces the strength and speed of movement and causes the patient to lose the ability to contract individual muscles. The cerebellum also regulates visceral output, but this function is less well understood.

Cerebellar granule neuron

Neurons are, by definition, postmitotic and therefore largely irreplaceable once they die. Neurons also communicate with each other at highly specialized structures called synapses, in which a neurotransmitter released from the presynaptic nerve endings. Synapses are often located at a relatively large distance away from the cell body, which is of considerable interest, since emerging data suggest synapses may be sites where neuronal apoptosis is often initiated (Mattson et al., 1998).

The cerebellar cortex of newborn rat, one of the best-studied regions of the developing brain, is a model well suited to identifying factors that control neuronal differentiation and apoptosis. In particular, cerebellar granule cells, which constitute the most abundant neuronal population in the mammalian CNS, have the advantages of a cell line without the drawbacks of transformed tumor cells. Granule cells have thus been widely used to investigate the neurotrophic or neurotoxic effects of various

factors and have provided crucial information regarding the basic molecular interplay of the cell death machinery (Vaudry et al., 2003).

Geldanamycin

Geldanamycin (GA), a naturally product, is produced by *Streptomyces hygroscopicus* var. *geldanus.*

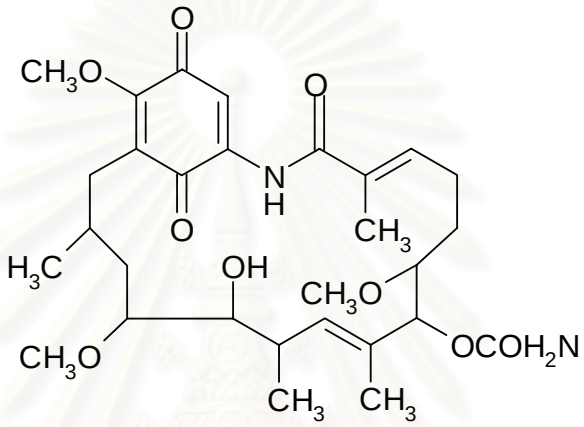

Fig. 1 Chemical structure of geldanamycin

Physical appearance: Yellow solid

Molecular formula: $C_{29}H_{40}N_2O_9$

Molecular weight: 560.6

Purity: > 95%

Solubility: soluble in dimethyl sulfoxide (DMSO) (10mg/ml)

Storage: Store, as supplied, at –20 ^{0}C for up to 1 year. Store solution at –20 ^{0}C for up to 3 months.

Pharmacological and toxicological properties of GA

GA, a benzoquinone ansamycin class antibiotic, possesses selective tumoricidal activity in preclinical models and is initially developed to be an antitumor agent. GA has been used as a tyrosine kinase inhibitor and is known to inhibit signaling by steroid hormone receptors, Src and Raf (Pratt, 1998). Previous studies showed that GA exerted inhibitory effects on cancer cells and transformed cells (Sasaki et al., 1979) and also inhibited DNA synthesis (Yamaki et al., 1982). GA has

been reported to associate specifically with the ATP/ADP-binding domain of heat shock protein 90 (HSP90) (Stebbins et al., 1997), resulting in dissociation of complexes between HSP90 and its partners and accelerating the degradation of these proteins, including the glucocorticoid and dioxin receptors, Raf, erbB2 and mutated p53 (Chen et al., 1997; Schulte et al., 1997). In addition, GA blocked intracellular translocation of the glucocorticoid receptor, Raf and mutated p53 (Dasgupta and Momand, 1997). These results suggest that functioning of HSP90 in signal transduction in term of stabilization and translocation of its partners could be inhibited by GA.

Antitumor effect of GA

The antitumor effect of GA is thought to result from antagonizing HSP90 chaperone family - HSP90. Binding of GA to the ATPase pockets of the chaperone proteins HSP90 causes the degradation or down-regulation of their substances involving proliferation, cell cycle progression, gene expression and apoptosis. Simultaneous combinatorial impacts on multiple tumorigenic client proteins and pathways may account for the mechanism underlying the anticancer effect of GA.

The antitumor effects of GA likely result from the ability to deplete cells of two broad classes of growth-regulatory signaling proteins (Stebbins et al., 1997):

1). Proto-oncogenic protein kinases, including the erbB2 and EGF receptor tyrosine kinases, the v-Src family of non-receptor tyrosine kinases and the Raf-1, and CDK4 Ser/Thr kinases whose overexpression, or otherwise deregulation, has been observed in diverse human cancers; and

2). The nuclear hormone receptor family, including the estrogen and androgen hormone receptors which can drive the growth of hormone-dependent cancers of the breast and prostate, respectively.

Protective effect of GA against focal cerebral ischemia

Recent *in vivo* study showed that geldanamycin decreased infarct volume, decreased brain swelling, decreased the number of cells with DNA fragmentation, improved behavioral outcomes and protected brain against cerebral ischemia via induction of heat shock proteins (Lu et al., 2002).

Anti-inflammatory effect of GA

Histamine, vascular endothelial growth factor, acetylcholine, estrogen, as well as fluid shear stress activate a mechanism that recruits heat shock protein 90 (HSP90) to the endothelial nitric oxide synthase (eNOS). The interaction between HSP90 and eNOS enhances the activation of the enzyme in cells of intact blood vessels leading to nitric oxide (NO) production and is important for the function of eNOS in response to growth factors, G-protein activation, and mechanotransduction (Pritchard et al., 2001). Bucci et al. (2000) showed that geldanamycin inhibited endothelium-dependent relaxation of the rat aorta, mesentery, middle artery, and inhibited carrageenan-induced mouse paw edema in a dose-dependent manner. In conclusion, geldanamycin, an inhibitor of HSP90-dependent signal transduction, displays anti-inflammatory effect in vivo implying that HSP90 is critical for pathways involved in carrageenan-induced paw edema. In addition, Shastry and Joyner (2002) showed that GA could attenuate NO-mediated vasodilation in human skin. Thus, the ability of GA to block NO release and reduce edema formation suggests a therapeutic rationale for specific inhibitors of HSP90 as potential anti-inflammatory drugs.

Inhibition of Huntingtin protein aggregation in vitro

Huntington's disease (HD) is a progressive neurodegenerative disorder with no effective treatment. Sittler et al. (2001) showed that treatment of mammalian cells with GA at nanomolar concentrations induced the expression of HSP40, HSP70 and HSP90 and inhibited HD exon 1 protein aggregation in a dose dependent manner.

Mediation of superoxide formation

GA contains a quinone group and such quinine-containing molecules are well known to have redox-active properties. Quinone reacts with flavin-containing enzyme cytochrome P-450 reductases and ascorbate to form superoxide (O_2^-). Notably, the cytotoxicity of the ansamycin antibiotics has been attributed to radical generation. Superoxide production by GA could be particularly important in the interpretation of studies in which it is used to study the function of eNOS. If GA is releasing either O_2^- or promoting the release of this radical, physiological response to endogenously produced $NO^{\cdot}$ may be lost because of the rapid reaction between $NO^{\cdot}$ and O_2^- rather than by specific inhibition of HSP90 (Dikalov et al., 2002). Diskalov et al. (2002)

showed that GA markedly increased both endothelial and vascular smooth muscle cell production of O_2^-. These studies indicated that this effect of GA is dependent on nitric-oxide synthase but probably involves the interaction of this drug with both small molecule reductants and flavin-containing enzymes. Finally, the data suggest that O_2^- released from GA rapidly reacts with $NO^\cdot$, reducing its ability to be trapped by Fe^{2+}(DETC).

Inhibition of platelet aggregation and disruption of membrane structure

Platelets are essential for hemostasis and normally circulate in a resting state. When exposed to agonists such as thrombin, ADP, and collagen, they can aggregate, change shape and secrete granule contents within seconds. Platelet activation by agonists leads to a change in the phosphorylation state of many platelet proteins on serine, threonine and/or tyrosine residues. In resting platelets, the state of protein phosphorylation must be actively balanced and ready to respond to agonist stimulation. In the study on human platelets, Suttitanamongkol et al. (2000) had found that GA inhibited platelet aggregation induced by ADP, thrombin and the thrombin-receptor-activating peptide. As a consequence, GA caused platelet plasma-membrane damage, detected by leakage of adenine nucleotides as well as serotonin. In conclusion, GA was able to disrupt membrane structure and inhibit platelet aggregation and caused major changes in protein phosphorylation in resting platelets as well as inhibiting increased phosphorylation in activated platelets.

Heat shock proteins (HSPs)

In all organisms proteins are continuously undergoing a folding process that is stabilized by a family of proteins known as chaperones. Molecular chaperones maintain the appropriate folding and conformation of proteins and are crucial in regulating the balance between protein synthesis and degradation. They have been shown not only to play a vital role in the cellular stress response but also to be important in regulating many important normal cellular functions, such as cell proliferation and apoptosis (Maloney and Workman, 2002).

Exposure of cells to a number of environmental stresses, including heat shock, alcohol, heavy metals, and oxidative stress results in the cellular accumulation of a number of chaperones, commonly known as heat shock proteins (HSPs). This effect is

mediated by the transcription factor heat shock factor 1 (HSF1) and is termed the ‘heat shock response’. Induction of HSPs protects the cell against the initial stress insult, enhances recovery and leads to maintenance of a stress-tolerant state. It has become clear, however, that certain HSPs may also play a major molecular chaperone role under normal, stress-free conditions by regulating the correct folding, degradation, localization and function of a relatively small but growing list of important cellular proteins (Maloney and Workman, 2002).

A number of multigene families of HSPs exist, with individual gene products varying in cellular expression, function and localization. They are classified according to molecular weight e.g. HSP70, HSP90 and HSP27. Exceptions to this nomenclature rule are a small subset of chaperones that were identified as glucose regulated proteins e.g. GRP94 and GRP75.

Several diseases in humans can be acquired as a result of protein misfolding. Hence the development of therapies which modulate the molecular chaperone machinery may prove to be beneficial. In some conditions, e.g. Alzheimer’s disease, prion diseases, and Huntington’s disease, misfolded proteins can cause protein aggregation resulting in neurodegenerative disorders. Also, misfolded proteins may result in loss of wild-type protein function, leading to deregulated molecular and physiological functions in the cell. With specific reference to the recent review, HSPs have been implicated in cancer. For example, there is evidence of differential expression of HSPs which may relate to the stage of tumor progression. As a result of the involvement of HSP90 in various critical oncogenic pathways and the discovery that certain natural products with anticancer activity are targeting this molecular chaperone, the fascinating new concept has been developed that inhibiting HSP function may be useful in the treatment of cancer (Maloney and Workman, 2002).

Heat shock protein 90 (HSP90)

HSP90 is a highly abundant protein which is essential for cell viability and it exhibits dual chaperone functions. It plays a key role in the cellular stress response by interacting with many proteins after their native conformation has been altered by various environmental stresses, such as heat shock, ensuring adequate protein folding and preventing non-specific aggregation. In addition, recent results suggest that HSP90 may also play a role in buffering against the effects of mutation, presumably

by correcting the inappropriate folding of mutant proteins and is required for the activation of several families of eukaryotic protein kinases and nuclear hormone receptors, many of which are protooncogenic and play a prominent role in cancer. However, HSP90 also has an important regulatory role under normal physiological conditions and is responsible for the conformation stability and maturation of a number of specific client proteins. These can be subdivided into three groups: steroid hormone receptors, serine/threonine or tyrosine kinases, and a collection of apparently unrelated proteins, including mutant p53. All of these proteins play key reguratory roles in many physiological and biochemical processes in the cell (Maloney and Workman, 2002)

HSP90: the target of the anti-tumor drugs geldanamycin

Specific inhibitors identified in the screening for natural substances that inhibit proliferation of tumor cells are important new tools for analysis of HSP90 function *in vivo* and *in vitro.* One of these, geldanamycin (GA), initially seemed to decrease the activity of certain cell-cycle kinases. It was therefore thought to be a kinase inhibitor. Surprisingly, however, the only cellular protein to which GA binds is HSP90 (Whitesell et al., 1994). The effect of GA on kinase activity is thus indirect and due to inhibition of HSP90, which affects kinase folding.

GA binds to the N-terminal domain of HSP90 (Stebbins et al., 1997; Grenert et al., 1997). Initially, studies suggested that the GA-binding pocket is the peptide-interaction site (Stebbins et al., 1997). Later, however, it was shown that the GA-binding site and ATP-binding site are identical and that GA is a competitive inhibitor. GA mimics the unusual kinked conformation of ATP extremely well; the complex formed is orders of magnitude more stable than that formed by ATP and HSP90.

Analysis of the effects of GA on steroid-receptor activation indicated that GA interferes with the binding of the partner protein p23 (Johnson, J. and Toft, D.O., 1995) and therefore blocks the chaperone cycle at the intermediate complex (Smith et al., 1995). Whether addition of GA leads to the release of non-native proteins from HSP90 or to prolonged binding is controversial. *In vitro* evidence suggests that, in addition to GA exerting effects on the association between p23 and HSP90, the compound directly influences the binding of non-native proteins to HSP90 (Scheibel et al., 1998).

Stress response mechanisms are essential for the maintenance of cellular integrity and viability. Diverse stress conditions converge to enhance the synthesis of HSPs, many of which function as molecular chaperones in protein biosynthesis, folding, assembly, translocation, and degradation (Bukau, B., and Horwich, A.L., 1998). In addition to preventing proteotoxic damage, HSPs also appear to be involved in antiapoptotic pathways (Buzzard et al., 1998). In animal models as well as in vertebrate cell culture models, overexpression of specific HSPs decreases cytotoxicity induced by different environmental stress conditions, including thermal and oxidative challenges, ischemia, or exposure to toxic chemicals. In the nervous system, HSPs are thought to play an important role in a variety of pathophysiological states, including neurodegenerative diseases, cerebral ischemia, epilepsy, and trauma.

Apoptosis and the nervous system

The life history of all neurons consists of several neurogenerative stages, including induction, differentiation, proliferation, migration, and formation of axonal pathways and synaptic connections, that eventually confer a specific physiological function to each neuron. However, in many parts of the CNS and PNS, roughly half of the neurons undergo an additional stage in which regression leading ultimately to death occurs. This relatively large loss of the neurons is a common feature in many types of neurons (motor, sensory, interneurons, autonomic, etc.), occurs in all vertebrates, and appears to have evolved as an adaptive mechanism during development of the nervous system (Oppenheim, 1991). It is known now that such large-scale cell death occurs in oligodendrocytes also (Barres et al., 1992). The general explanation for this phenomenon is that the survival of developing vertebrate neurons depends on specific neurotrophic factors secreted by the target cells that the neurons innervate. There is excellent experimental proof for the above hypothesis and several neurotrophic factors called “neurotrophins”, like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins (NT)-3/4/5 have been shown to promote the survival of specific developing neurons. In addition, the developing neurons also require signals from other neurons that innervate them, some require specific hormones, and perhaps some require signal from neighboring glial cells as well (Raff et al., 1993). Thus, the survival of neurons depends on a complex interplay of several factors, and any imbalance in these inputs may lead to cell death. The beneficial effect of this mechanism is that although many types of neurons are

produced in excess, only a portion of them get sufficient neurotrophic support for their survival, and the rest die facilitating appropriate neuron-target cell innervation.

The cell death phenomenon, apart from being an important feature in the development of the nervous system, appears also to be a cause for many neurodegenerative diseases. Many neurological diseases are characterized by the gradual loss of specific sets of neurons and result in disorders of movement and CNS function. Such diseases are Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa, several forms of cerebellar degeneration, spinal muscular atrophy, Alzheimer's disease amd Huntington's disease. Aging, which may be considered as the terminal phase in the development of an organism, may also be a manifestation of specific neuronal loss by cell death. Several fectors, such as oxidative stress, excitatory toxicity, inappropriate Ca^{2+} homeostasis, mitochondrial dysfunction, and lack of sufficient neurotrophic factors, have all been shown to contribute to the above-mentioned disorders. The available evidence indicates that the survival of neurons and their death are highly regulated, a dynamic events that depend on a number of internal and external factors, with vital consequences to the well-being of an organism.

Kerr et al. (1972) described two types of cell death. One, called "cell necrosis," results from injury and causes inflammation. The other, called "apoptosis," is the normal developmental type with several distinct characteristics. The term apoptosis is derived from Greek roots meaning "dropping of leaves off a tree." A number of features defines apoptosis and differentiates it from necrosis, although some features are also overlapping with necrosis. A principle distinguishing factor is that apoptotic cell death is an "active" process, for which protein synthesis is required. During this process, there is consecutive activation of a variety of otherwise dormant pathways. Thus, apoptosis may be conceived of as "programmed active cell death," as opposed to "passive cell death." This distinction has led to the denotation of apoptosis as "cell suicide." Another significant feature of apoptosis is that it usually involves isolated single cells, with topographically and temporally dispersed cell death within an organ. In contrary, necrosis often occurs in a regional group of cells at a particular point in time.

Pathological features of apoptosis include a number of attributes morphologically and biochemically distinguishing the process from necrosis. Most of

these are at the single cell level, although necrosis is usually a more massive phenomenon, affecting whole tissues, while apoptosis usually involves single cells, as mentioned above. Necrosis involves substantial cell swelling, with membrane breakdown and leakage of cell contents extracellularly. Apoptosis classically involves involutional change, with cell shrinkage being the characteristic shape change. During necrosis, the cytoplasmic organelles, such as mitochondria and lysosomes, usually swell and lyse, while in apoptosis there is contraction, with formation of “apoptotic” bodies, or small dense vesicular units that are phagocytized by macrophage without an inflammatory response. Apoptosis involves plasma membrane changes, clearly visualized in culture, but also recognizable in vivo, with marked cytoplasmic blebbing and involution. Nuclear changes of necrosis consist of disruption of nuclear membrane integrity; nuclear contents may show histopathological fading or intensifying, sometimes with fragmentation of the nucleus. By contrast, in apoptosis, the characteristic nuclear changes involve clumping of chromatin, sometimes leading to a “spoked wheel” appearance. A prominent molecule hallmark of apotosis is a specific pattern of internucleosomal fragmentation of DNA. This leads to the appearance on agarose gel electrophoresis bands differing from their neighbors in molecular weight multiples of about 180 base pairs. In necrosis, there is also DNA breakdown, but it is apparently more diffuse and random, leading to a “smear” on gel electrophoresis rather discrete multiple bands (Lawrence et al., 2000). Biochemical changes that may distinguish apoptosis and necrosis in neurons are beginning to be identified. Neurons undergoing apoptosis exhibit: rapid increases in prostate apoptosis response-4 (Par-4) protein levels (Guo et al., 1998; Chan et an., 1999); translocation of one or more members of the Bcl-2 protein family to mitochondrial membranes (Putcha et al., 1999); mitochondrial membrane depolarization and release cytochrome C into the cytoplasm (Neame et al., 1998): activation of one or more members of the caspase family of cystein proteases (Chan and Mattson, 1999); loss of plasma membrane phospholipid asymmetry which manifests in the presence of phosphatidylserine on the cell surface (a signal for engulfment by microglia); and nuclear DNA fragmentation (Kruman et al., 1997; Mattson et al., 1998). These changes are typically not observed in neurons undergoing necrosis.

Induction of apoptosis

A variety of compounds and experimental conditions have been found to induce apoptosis in cells of the nervous system, and these may be classified as shown in Table 1

Table 1. Inducers of apoptosis in cells of the nervous system

Group	Conditions/agent	Cell type
A	Neurotrophic factors	
	Withdrawal of NGF from culture medium	Synpathetic neurons
		Chick embryo sensory neurons
		Motoneurons
	Withdrawal of serum from culture medium	Cortical neurons
		Cerebellar granule cells
		Proliferating cell line of neuronal origin
	Insulin deprivation	Cerebellar granule cells
	Glial-derived neurotrophic protein S100β	Astrocyte-neuron cocultures
	Basic fibroblast growth factor	Chick embryonic neural retinal cells
B	Changing from a high K^+ (25 mM) to a low K^+ (5 mM) medium	Cerebellar granule cells
		Cerebellar neurons
C	Addition of modulators of protein phosphorylation	Cortical neurons
	protein kinase inhibitor: staurosporine	Embryonic chick cerebral neurons
		Cerebellar granule neurons
		Embryonic astrocyte culture
		Sympathetic neurons
		Hippocampal neurons
D	DNA-damaging agents and nucleosides	
	Topoisomerase-1 inhibitor: camptothecin	Cortical neurons
	Topoisomerase-2 inhibitors:tenoposide and mitoxantrone	Sympathetic neurons
		Sympathetic neurons
	Etoposide	Cultured neurons
	Arabinonucleosides of adenine, cytosine, guanine, and thymidine	Sympathetic neurons
	2-Deoxyadenosine	Cerebellar granule cells
	2-Chloroadenosine	Chick embryonic sympathetic neurons
	N-Methyl-N'-nitro-N-nitrosoguanine	Cultured astroglial cells

Group	Conditions/agent (cont.)	Cell type
E	Effectors of calcium homeostatis	
	Calcium ionophores	Central neural cell line
	Endoplasmic reticular Ca2+-ATPase	Cultured cortical neurons
	inhibitor :thapsigarin	GT1-7 murine hypothalamic cell line
F	Neurotransmitters	
	Excitatory amino acids	Cultured neurons
	Glutamate	Freshly isolated cortical neurons
		Hippocampal neurons
		Cultured cortical neurons
	NMDA/quisqualate	Cerebellar granule cells
		Developing brain
	Dizocilpine (injected into animals)	Cultured neurons
	Kainic acid (injected into animals)	Cerebellar granule cells
		Hippocampal entorhinal sensory cortex
		Hippocampus inner cortical layers
	Dopamin	Primary striatal cultures
		Olfactory neuronal cell line
G	Peptides and proteins	
	Amyloid β-peptide	Cultured cortical neurons
	Recombinant HIV-1 gp120	Cultured hippocampal neurons
	Soluble macrophage proteins	Cortical neurons
		Cultured hippocampal neurons
H	Oxidative stress	
	Ischemia (artery occlusion, reperfusion)	Cerebral neurons
	Hypoxia	Hippocampal neural cell line
		Neurons cocultured with astrocytes
	High oxygen (50% oxygen atmosphere)	Cultured hippocampal neurons
	Hydrogen peroxide	Neuroblastoma
	Glutamate-induced glutathione depletion	Cortical neurons
	3-Hydroxykynurenine	Cultured striatal neurons
	Inhibition of SOD	Spinal cord organotypic cultures
I	NO	
	NO donors: sodium nitroprusside and S-nitroso-N-acetylpenicillamine	Human neuroblastoma cell line

Group	Conditions/agent(cont.)	Cell type
	NO-releasing compound	Human neuroblastoma cell line
	Treatment with interferon-γ plus interleukin-1β	Primary human neuronal/glial mixed culture
J	Lipids	
	Ceramide	Primary culture of mesencephalon
		Cerebellar granule cells
		Astrocyte culture
	Retinoic acid	Hippocampal neurons
		Embryonic stem cell line
	Lysophosphatidic acid	Embryonic carcinoma cells
	Phosphatidyllinositol 3-kinase inhibitors	Cultured hippocampal neurons
	Cholesterol	Cerebellar granule cells
		Cerebellar neurons
K	Irrdiation	
	Ultraviolet	Cultured cerebellar neurons
	χ irradiation	Cultured hippocampal neurons
	γ irradiation	3-day-old rat brain neurons
		Fetal rat brain
	Ionizing radiation	Cerebellar granule cells in vivo
		Cultured cerebellar neurons
		Cultured hippocampal neurons
L	Neurotoxicants	
	MPP^+	Cerebellar granule cells
		Substantia nigra
	MDMA	Human serotonergic JAR cells
	Cocaine	Neuronal cultures
	Ethanol	Cerebellar granule cells
	Methyl mercury	Cerebellar neurons
	Dibutyryl cyclic AMP	Neuroblastoma B50 and B104 cells
	Cycloheximide	Neuroblastoma B50 and B104 cells
	Dimeric interleukin-2	Cultured oligodendrocytes
	Colchicine	Cerebellar granule cells

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Low potassium-induced apoptosis

The mammalian nervous system is critically dependent on trophic support for proper development and survival (Barde, 1989). Among the molecules shown to influence neuronal differentiation and survival are the neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT3 and NT4), the fibroblast growth factors (FGFs), ciliary neurotrophic factor (CNTF), insulin, and the insulin-like growth factors (IGFs) (Knusel et al., 1990).

In culture, neuronal survival can also be supported by various agents in the absence of any neurotrophic factors. One of these agents is K^+. Depolarization by high levels of extracellular K^+concentration ($[K^+]$) promotes survival of several types of neurons in vitro (Koike et al., 1989; Gallo et al., 1990; Franklin and Johnson, 1992). Besides supporting neuronal survival, high K^+ also influence neuronal development and phenotypic characteristics (Chalazonitis and Fishbach, 1980; Ishida and Deguchi, 1983; Resink et al., 1992). Although the ability of high $[K^+]$ to promote neuronal survival is well established, the mechanism by which it acts is not fully understood; $[K^+]$ may exert its rescuing activity through activation of dihidropyridine sensitive calcium channels (Gallo et al., 1987; Koike et al., 1989).

Among the various cell types known to be dependent on high K^+ for survival in vitro are cerebellar granule neurons (Lasher and Zagon, 1972; Levi et al., 1984; Gallo et al., 1990), which constitute the most abundant neuronal population in the mammalian brain. The natural neurotrophic factor, if it exits, that promotes survival of cerebellar granule neurons has not yet been identified. D'Mello et al. (1993) found that lowering the potassium concentration induced apoptosis in these neurons. The death process is dependent on the synthesis of both new RNA and proteins. Insulin-like growth factor I and cyclic AMP can protect the granule neurons from apoptosis in low K^+. Wood et al. (1993) found the in vivo correlate of apoptosis in cerebellar granule cells - naturally occuring DNA fragmentation in the granular layer of newborn rat cerebellum. Dissociated cerebellar granule neurons from neonatal rats (postnatal day 8) differentiate acquiring morphological, biochemical, and electrophysiological characteristics of mature neurons (Lasher and Zagon, 1972; Hockberger et al., 1987; Cull-Candy et al., 1988;Gallo et al., 1990) and serve as an excellent model to study the molecular mechanism of PCD. In several studies on cerebellar granule cells

(D'Mello et al., 1993, 1998; Galli et al., 1995; Miller et al., 1996; Nath et al., 1996; Taylor et al., 1997) and cerebellar neurons (Kunimoto, 1994), changing [K^+] from 25 mM to 5 mM in the culture medium resulted in neuronal apoptosis.

When cerebellar granule neurons obtained from postnatal rats are cultured in medium containing serum and depolarizing level of K^+ (25 mM), they can survive and mature *in vitro*. After culture and maturation in HK medium, lowering the K^+ concentration (5 mM) in the medium without serum induced death of cultured granule neurons. This LK-induced cell death occurs via apoptosis, as demonstrated by the typical hallmarks of apoptosis such as membrane blebbing, nuclear and cytoplasmic condensation, and DNA fragmentation (D'Mello et al., 1993; Galli et al., 1995; Schulz et al., 1996). An alteration of reactive oxygen species (ROS) metabolism and cellular redox state have also been implicated as mediators of CGC apoptotic death (Simonian and Coyle, 1996; Atlante et al., 1998). A sequential requirement for new mRNA formation, protein synthesis, caspase activation, and finally ROS overproduction during CGC apoptosis has been demonstrated (Schulz et al., 1996). Free radicals can act either as effectors of DNA, lipid, and protein damage or as signaling molecules via redox-sensitive cellular factors, such as nuclear factor-κB (NF-κB) (Bredesen, 1995). Other events during potassium deprivation-induced apoptosis include synthesis and phosphorylation of c-Jun (Watson et al., 1998), inactivation of phosphatidylinositol-3 (PI-3) and Akt kinase (D'Mello et al., 1997; Shimoke et al., 1997; Skaper et al., 1998) and BAD dephosphorylation (Gleichmann et al., 2000).

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CHAPTER III

MATERIALS AND METHODS

Experimental Animals

Postnatal days 8, Wistar rat (both sexes) were used in this study. They were obtained from time-pregnant rats of embryonic day 18 that purchased from the National Laboratory Animal Center, Salaya, Mahidol University, Bangkok. All pregnant rats were housed in stainless steel cage until delivery and rat pups were kept with their mother until 8-day old. They were freely accessed food pellets (C.P. rat fed, Pokaphand Animal Fed Co. Ltd., Bangkok, Thailand) and water in the Central Animal House, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Chemicals

1. n-Butanol [BDH]
2. Butylated hydroxytoluene [sigma]
3. Calcium chloride [Ajax chemicals]
4. Cytosine arabinoside [Sigma]
5. Dimethyl Sulfoxide (DMSO) [BDH]
6. 5-5’ dithiobis-(2-nitrobenzoic acid) (DTNB) [Sigma]
7. DNase I [Sigma]
8. Dulbecco’s Modified Eagle’s Medium (DMEM) [Sigma]
9. Dulbecco’s Modified Eagle’s Medium (DMEM) without phenol red [Sigma]
10. DMEM nutrient mixture F-12 HAM [Sigma]
11. Dulbecco’s Phosphate-Buffered Saline (DPBS) [Sigma]
12. Equin Serum [Hyclone]
13. Ethanol [Merck]
14. Ether [Labscan]
15. Fetal Bovine Serum (FBS) [Hyclone]
16. Glutathione, reduced form [Sigma]
17. Glutathione reductase [Sigma]
18. Hank’s Balance Salt Solution [Sigma]

19. HEPES [Sigma]
20. Hydrochloric acid [Merck]
21. Insulin [Sigma]
22. In vitro Toxicology Assay Kit (Lactate Dehydrogenase Based) [Sigma]
23. Magnesium chloride [Merck]
24. Magnesium sulphate heptahydrate [BDH]
25. Methanol [BDH]
26. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) [Sigma]
27. β-Nicotinamide adenine dinucleotide phosphate, reduced form(β-NADPH) [Sigma]
28. Penicillin G [Sigma]
29. Phosphotungstic acid [Sigma]
30. Poly-D-Lysine Hydrobromide (MW 15,000-30,000) [Sigma]
31. Potassium chloride [Ajax chemicals]
32. Progesterone [Sigma]
33. Putrescine [Sigma]
34. Pyruvic acid sodium salt [Fluka]
35. Sodium selenite [Sigma]
36. Sodium bicarbonate [BDH]
37. Streptomycin sulfate [Sigma]
38. Sulfosalicylic acid [Sigma]
39. Sulfuric acid [BDH]
40. Thiobarbituric acid [BDH]
41. Transferrin [Sigma]
42. Tripsin [Sigma]
43. Triton X-100 [Sigma]
44. Trolox (a water-soluble vitamin E) [Sigma]
45. 0.04% Trypan blue [Sigma]

Application of Geldanamycin and Trolox

Geldanamycin (GA) was kindly provided by Dr. Khanit Suwanborirux (Department of Pharmacognosy, Chulalongkorn University).

Both geldanamycin and trolox solutions dissolved in DMSO were directly added to culture medium with a final concentration of DMSO less than 0.5% v/v.

Culture Media

All culture media were obtained from commercial sources in powder form. They were prepared by dissolving ingredients in nonpyrogenic distilled water followed by filtered sterilization through a 0.22 μm Millipore (Bedford, MA) filter. They were adjusted to pH 7.4 and kept at 4^{o}C until used.

Dulbecco's Modified Eagle's Medium (DMEM, high glucose) was supplemented with 99 μg/ml sodium pyruvate, 3.7 mg/ml sodium bicarbonate, 54 μg/ml penicillin G sodium, 90 μg/ml streptomycin sulfate and 10%(v/v) fetal bovine serum (FBS).

Serum-free DMEM was composed of normal DMEM without phenol red supplemented with 30 μg/ml human transferrin, 5 μg/ml insulin, 100 μM putrescin, 20 μM progesterone and 30 nM sodium selenite.

Instruments

1. Adjustable pipette: 10-100 μl [Nichiyo], 200-1000 μl, 1-5 ml [Labsystem]
2. Aluminium Foil [Tops]
3. Bunsen bumer
4. Carbon dioxide incubator [Forma Scientific]
5. Cell culture dish : diameter 35 mm, diameter 100 mm [Nunc]
6. Cell strainer : 40 μm Nylon [Becton Dickinson]
7. Centrifuge [Kokusan]
8. Conical tube : 15 ml, 50 ml [Nunc]
9. Disposible glass pasteur pipettes : 230 mm [Volac]
10. Fluorescence spectrophotometer FS 777 [Jasco]
11. Glass bottle: 100 ml, 500 ml, 1000 ml [Schott Duran]
12. Hemocytometer (Depth 0.100 mm)[Improved Neubauer]
13. Inverted microscope, Axiovert 135 [Zeiss]
14. Laminar air flow hood [Hepaco]
15. Latex Free Syringe : 10 ml [Becton Dickinson]

16. Microliter pipette : 10-100 μl [Nichiryo]
17. Microliter pipette : 200-1000 μl, 1-5 ml [Labsystems]
18. Microplate reader [Anthos htl]
19. Needle-18 G
20. pH meter [Beckman Instrument]
21. Surgical equipments
22. Sterile Millex – GV (0.22 μm filter unit) [Millipore]
23. Sterivex – GS (0.22 μm filter unit with filling bell) [Millipore]
24. Sonicator [Elma]
25. Vortex mixer [Clay adams]
26. Water bath [Thelco]
27. 24-well cell culture plate [Nunc]
28. 96-well microtiter plate [Nunc]

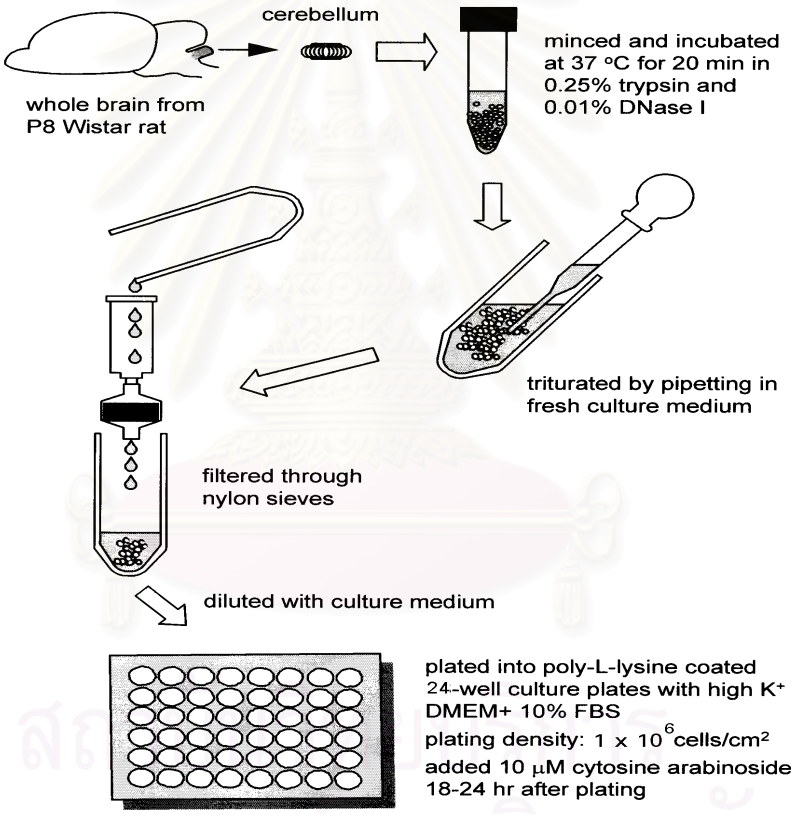
Methods

Culturing of cerebellar granule neurons

Preparation of primary cultures of cerebellar granule neurons was carried out as described in details by Unchern et al (1997) using pooled cerebella of postnatal rats. Cerebella were removed from the brains of 8-day-old Wistar rats, a time when many of the granule neurons were still at an early post-mitotic stage of differentiation. After removal of the meninges the tissue was cut into pieces and incubated for 20 min at 37 ^{0}C with 0.25% trypsin and 0.01% DNase I in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution. The incubation was terminated by the addition of heat-inactivated horse serum and tissue fragments were centrifuged at 3,000 rpm for 10 min. The tissue pellet was gently rinsed and resuspended in high K^{+} (25 mM KCl) DMEM containing 10% FBS. The single cells were dissociated by gently passing the suspension through a 10-ml plastic pipette tip and then a flame-polised Pasteur pipette tip. The cell suspension was filtered through two sheets of nylon net (50 μm-mesh) to remove cell lumps. The cells were counted on hemocytometer using trypan blue exclusion and diluted as appropriate with high K^{+} DMEM containing 10% FBS and plated on poly-D-lysine (100 μg/ml) coated plates at a density of 1 x 10^{6} cells/cm^{2} in 24-well plates. The cultures were grown in a humidified 5% CO_2- 95% air atmosphere at 37 ^{0}C. At 18-24 hr after plating, cytosine arabinoside was added to the medium to a final

concentration of 10 μM; this curtails the number of astrocytes that develop in the cultures. One-third of volume of each well was replaced with fresh high K^+ (25 mM KCl) DMEM containing 10% FBS on day 4. Cultures were used on day 8 at which the medium was changed to serum-free low K^+ (5 mM KCl) DMEM or serum-free high K^+ (25 mM KCl) DMEM with/without phenol red plus desired concentrations of geldanamycin or trolox.

Fig. 2 Preparation of cerebellar granule neuronal cultures



Treatment of primary cerebellar granule neuronal cultures

Effects of geldanamycin on cultured rat cerebellar granule neurons

Cultures were exposed to geldanamycin at the concentrations of 0.01, 0.05, 0.1, 0.5, and 1.0 μM for 12, 24, 48, and 72 hours in serum-free high K^+ (25 mM KCl) DMEM containing N2 supplements. Cell viability was measured by MTT reduction assay and LDH release assay to determine the effects of time and concentration of exposure with geldanamycin.

Time course of low K^+-induced injuries on cultured rat cerebellar granule neurons

Cultured neurons were switched from culture medium containing 10% fetal bovine serum and 25 mM KCl to serum –free DMEM containing 5 mM KCl. Cultures were exposed to serum-free low K^+ (5 mM KCl) DMEM containing N2 supplements for 6, 12, 18, 24, 36, and 48 hours. Cell viability was measured by MTT reduction assay and LDH release assay to determine the time course of low K^+-induced injuries.

Effects of coexposure with geldanamycin or trolox on low potassium-induced injuries in cultured rat cerebellar granule neurons

Cultures were incubated with geldanamycin at the concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 μM or trolox at the concentrations of 10, 25, 50, 75, and 100 μM for 24 hours in serum-free low K^+ (5 mM KCl) DMEM containing N2 supplements. After the incubation period, MTT reduction assay and LDH release assay were done. In certain experimental conditions, TBARS assay and glutathione assay were also performed.

Effects of preexposure with geldanamycin or trolox on low potassium-induced injuries in cultured rat cerebellar granule neurons

Cultures were first incubated with geldanamycin at the concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 μM or trolox at the concentrations of 10, 25, 50, 75, and 100 μM for 6, 12, 24, 48, 72 hours in serum-free high K^+ (25 mM KCl) DMEM containing N2 supplements. After the incubation period, cultured cells were switched to fresh serum-free low K^+ (5 mM KCl) DMEM containing N2 supplements for 24 hr.

Then MTT reduction assay and LDH release assay were done. In certain experimental conditions, TBARS assay and glutathione assay were also performed.

Analysis of cell viability

MTT reduction assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay is a simple non-radioactive colorimetric assay to measure cell cytotoxicity, proliferation or viability. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring by mitochondrial dehydrogenase of living cells. The resulting intracellular purple formazan can be measured by spectrophotometry after extraction by dimethyl sulfoxide. This assay is used to measure changes in cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, MTT reduction decreases, reflecting the loss of cell viability.

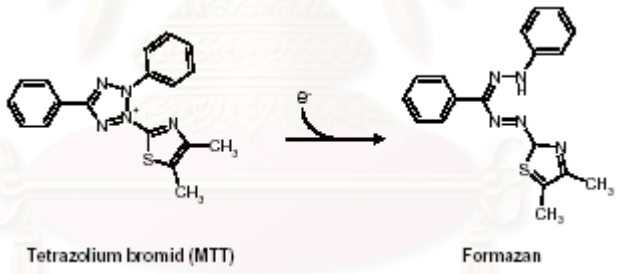


Fig. 3 Structure of the yellow water soluble MTT and its dark blue insoluble formazan

In this study, the MTT reduction assay technique was modified from Mossmann, 1983 and Hansen et al., 1989. To determine the direct influence of screening substances on the MTT-tetrazolium reduction, these substances or extracts were incubated in free system and the formation of formazan was determined. 16 µl of the MTT dye solution [5mg/ml in phosphate-buffered saline (PBS) pH 7.4,MTT Sigma] was added to cell culture medium in each well (final concentration were 100µg/ml). The assay was incubated in CO_2 incubator at 37^{o}C for 1 hr and the medium in each well was aspirated off without disturbing the formazan precipitate.

Then 400 μl of DMSO was added to each well in order to solubilize the formazan crystals. Following through formazan solubilization, 200 μl aliquots of soluble formazan were transferred to a-96well microtiter plate. The amount of MTT formazan product was determined by measuring absorbance with a microplate reader (Anthos htl) at a test wavelength of 570 nm and a reference wavelength of 655 nm. The results were expressed in term of the percentage of absorbance in control cultures.

Lactate dehydrogenase (LDH) release assay

Lactate dehydrogenase release assay is a simple colorimetric assay for the quantitation of cytotoxicity/cytolysis based on the measurement of LDH activity released from damaged cells. Lactate dehydrogenase (LDH) leakage through the cell membrane is routinely used as an indicator of damage to plasma membrane integrity. An increase in LDH leakage into the extracellular environment indicates a loss of membrane integrity, and is interpreted as a cytotoxic effect.

Cell viability was determined by assaying the medium from each well for lactate dehydrogenase (LDH) activity using an in vitro toxicology assay kit (Takara, Japan) according to the manufacture's instructions. Briefly, medium LDH was assayed by pipetting 100 μl of culture medium from each well into a 96-well microtiter plate. Cellular LDH was measured by carefully aspired off the remaining culture medium and solubilized cells with 1 ml of 0.5% Triton X-100 in PBS after which 100 μl aliquots were pipette into a 96-well microtiter plate. The reaction was started by addition of 50 μl of lactate dehydrogenase assay mixture into each well. The reaction mixtures were protected from light and left at room temperature for 30 min. The reaction was terminated by adding 50 μl of 0.5 N HCl into each well. The light absorbance in each well was measured at 490-655 nm with a Biorad 3550 dual wavelength microplate reader. The LDH release which reflects cell death was presented as persentage of total LDH activity by the following formula:

$$\%\text{LDH release} = \frac{\text{LDH activity in medium x 100}}{\text{LDH activity in medium + LDH activity in cells}}$$

In most cases, comparative LDH release in test conditions was expressed as the percentage of that in control conditions.

Glutathione assay

Glutathione (GSH) is the most abundant thiol (SH) compound in animal tissue, plant tissues, bacteria and yeast. GSH plays many different roles such as protection against reactive oxygen species and maintenance of protein SH groups. During these reactions, GSH is converted into glutathione disulfide (GSSG: oxidized form of GSH). GSH measurement can be done in plasma, tissue sanples and cultured cell.

DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)), known as Ellman's Reagent, was developed for the detection of thiol compounds. In 1985, Dr. Anderson suggested that the glutathione recycling system by DTNB and glutathione reductase created a highly sensitive glutathione detection method. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in a sample solution can be determined by the measurement at 450 nm absorbance (Tietze, 1969; Baker et al., 1990). GSH is generated from GSSG by glutathione reductase, and reacts with DTNB again to produce 2-nitro-5-thiobenzoic acid. Therefore, this recycling reaction improves the sensitivity of total glutathione detection (Fig. 5).

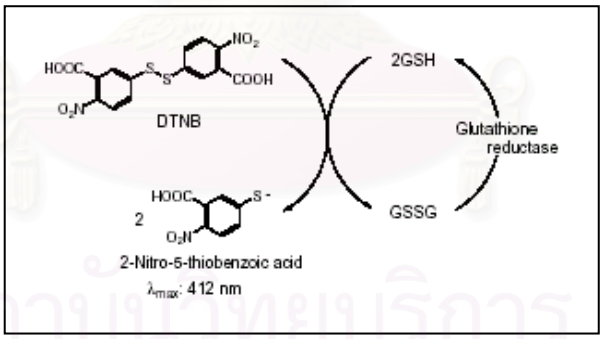


Fig. 4 Principle of total glutathione quantification kit

In this study, cells were washed twice in cold PBS, dissolved in 150 μl of 1% w/v sulfosalicylic acid and left on ice for 10 min. Then, two wells of samples were pooled, transferred to Eppendorf tubes and centrifuged at 13,000 x g for 5 min at 4^{o}C. The supernatant was analyzed for total glutathione. A 20 μl aliquot of supernatant was added into the well of a 96-well microtiter plate and adjusted to 100 μl with 0.1 M phosphate buffer containing 1mM EDTA (pH 7.5). The reaction was initiated by addition of 100 μl of reaction mixture [25 μl of 0.15 mM 5,5'-dithiobis (2-

nitrobenzoic acid), 25 μl of 0.2 mM NADPH and 50 μl of 1 U GSH reductase in 0.1 M sodiumphosphate buffer (pH 7.5)]. The formation of 2-nitro-5-benzoic was monitored at 30-s intervals for 10 min at 450/620 nm by using a microplate reader [Anthos htl]. The slope of the initial rate of reaction was used for calculating GSH content from a standard curve obtained by plotting known amount of GSH (25-150 μM). The cellular content of GSSG was typically less than 2 % of the GSH level and was not considered.

Thiobarbituric acid reactive substances (TBARS) assay

The sensitivity of measuring thiobarbituric acid reactive substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress (Yagi et al.,1998). Malondialdehyde (MDA), an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for assessing lipid peroxidation. MDA forms a 1:2 adduct with thiobarbituric acid (TBA) and produces a colored MDA-TBA adduct which can be measured by fluorometry or spectrophotometry.

TBARS was measured using a technique modified from Ohkawa et al. (1979) and Storch et al. (2000). Briefly, cultured cells were washed three times with Hank's balanced salt solution (HBSS) and lysed with 160 μl of 2% sodium dodecyl sulfate for 30 min.Cell lysates from six cell culture wells were pooled, transferred to glass tube and adjusted with 2% sodium dodecyl sulfate to 1 ml. The lysates were added serially with 50 μl of butylated hydroxytoluene (4% in ethanol), 1 ml of phosphotungstic acid (10% in 0.5 M sulfuric acid) and 1.5 ml of thiobarbituric acid (0.7%). The mixtures were incubated at 95^{o} C for 60 min, cooled by tap water, extracted with 4 ml of n-butanol and centrifuged at 3,500 rpm for 10 min. After centrifugation, the fluorescence of the n-butanol layer was measured at 515 nm excitation and 553 nm emission with Jasco FS 777 spectrofluorometer. Results are expressed in term of percentage control.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) from groups of 6-8 samples. Differences among experimental groups were assessed by one-way

analysis of variance (ANOVA) followed by Scheff's test for multiple comparisons. Statistical significance was considered with $P < 0.05$.

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CHAPTER IV

RESULTS

1. Effects of geldanamycin on cell survival of cultured cerebellar granule neurons

After the exposure of cerebellar granule neurons to geldanamycin (GA) at different concentrations (0.01-1.0 μM) for various time intervals (12-72 hr), it appeared that low concentrations of GA (0.01-0.1 μM) produced no significant changes in cell metabolic activity. At concentrations higher than 0.50 μM with 12, 24, 48, and 72 hr of incubation, GA markedly decreased mitochondrial metabolic activity (Fig. 5). In addition, low concentrations of GA (0.01-0.1 μM) produced no significant neuronal cell death within the first 24 hr of exposure. However, after 24 hr of exposure, neuronal injuries and death were apparent with concentrations of GA higher than 0.50 μM. It was notable that with long-term exposure (after 72 hr of incubation), signs of neuronal cell death were also apparent with GA concentration of 0.05 μM (Fig. 6). Therefore, it was evident that GA might be cytotoxic to cultured cerebellar granule neurons if the concentrations or time of exposure were increased up to a certain limit.

2. Time course of low K^+-induced injuries in cultured cerebellar granule neurons

To investigate the effect of low K^+, cultured neurons were allowed to develop for 8 days in serum-containing medium and 25 mM K^+ (high K^+). The mature neurons were then shifted to serum-free medium containing 5 mM K^+ (low K^+). Control cells were maintained in serum-free medium with 25 mM K^+. Cell death was not detectable within 6 hr of K^+ deprivation but < 50% of the cells were viable at 24 hr. Significant decrease of MTT reduction was observed as early as 12 hr of incubation and continued to decrease to the level of < 40% by 48 hr (Fig. 7). In addition, LDH release into the culture medium was low within 12 hr of K^+ deprivation but increased to ≈ 50% by 24 hr and thereafter increased up to 48 hr of incubation (Fig. 8). Taken together, the results of these two assays indicated that neuronal death began after ~ 12 hr of K^+ withdrawal and proceeded rapidly within the next 12 hr and after 24 hr of K^+ deprivation, ~ 50% of the neurons had lost their viability. Therefore, as considered

from the time course of low K^+-induced injuries in cultured cerebellar granule neurons, 24 hr of incubation with low K^+ was used as an insulting condition in subsequent experiments of this study.

3. Effects of cotreatment with geldanamycin on low K^+-induced injuries in cultured cerebellar granule neurons

After the co-exposure of cultured cerebellar granule neurons with GA, at different concentrations (0.01-0.10 μM), and low K^+ medium for 24 hr, GA aggravated the suppression of cell metabolic activity (to ~ 40-45% of control, $p < 0.05$) (Fig. 9). In addition, GA had no protective effect on neuronal death induced by low K^+ but instead significantly increased neuronal cell death (Fig. 10). A similar inability to prevent low K^+-induced neuronal death was also seen with trolox. The co-incubation with 100 μM trolox was not effective in preventing neurons from low K^+-induced injuries.

4. Effects of pretreatment with geldanamycin on low potassium-induced injuries in cultured cerebellar granule neurons

After the pre-exposure of cultured cerebellar granule neurons with GA at different concentrations ranging from 0.01-0.10 μM for 6, 12, 24, 48, and 72 hr of incubation, neurons were shifted to serum-free low K^+ medium (5 mM KCl) for 24 hr. Control cells were maintained in serum-free medium with high K^+ (25 mM KCl). It appeared that after 6 hr pre-exposure to 0.01-0.10 μM of GA, the MTT reduction increased gradually up to 72 hr of incubation. At low concentrations of GA (0.01-0.025 μM) with short incubation periods (6-12 hr), GA showed a little effect on neuronal metabolic activity. However, after 24-72 hr pre-exposure to GA at concentrations higher than 0.05 μM, GA had a marked effect on neuronal metabolic activity as compared to neurons in serum-free medium containing 5 mM K^+ ($p < 0.05$) (Fig. 11-15). In addition, pre-exposure of cerebellar granule neurons to low concentrations of GA (0.01-0.025 μM) for 6-12 hr had a little but statistically significant effect on neuronal survival as compared to neurons in serum-free medium containing 5 mM K^+ (Fig. 17-18). With 24 hr pre-exposure to GA at concentrations higher than 0.025 μM, cell death caused by low K^+ was significantly prevented (Fig. 19-20). However, 72 hr incubation with 0.01-0.10 μM GA failed to significantly

inhibit death of granule neurons that caused by K^+ deprivation (Fig. 21). Taken together, these biochemical assays indicate that GA exerts a concentration-dependent protective effect on low K^+-induced injuries to cultured cerebellar granule neurons. Apparently, the maximal magnitude of protection may be obtained from 24 hr pre-exposure to 0.10 μM GA (Fig.16, 22).

5. Effects of cotreatment with trolox on low potassium-induced injuries in cultured cerebellar granule neurons

After the co-exposure of cultured cerebellar granule neurons with trolox at different concentrations (10-100 μM) and serum-free low K^+ medium (5 mM KCl) for 24 hr, it was apparent that trolox aggravated the suppression of cell metabolic activity (to > 50% of control, $p < 0.05$) (Fig. 23) and significantly increased neuronal cell death (Fig. 24).

6. Effects of pretreatment with trolox on low potassium-induced injuries in cultured cerebellar granule neurons

After the pre-exposure of cultured cerebellar granule neurons with trolox at different concentration ranging from 10-100 μM for 24, 48, 72 hr and then switching to serum-free low K^+ medium (5 mM KCl) for 24 hr, trolox sinificantly increased mitochondrial metabolic damage and neuronal cell death (Fig. 25-30). These results, in conjunction with the results of cotreatment with trolox on low potassium-induced injuries in cultured cerebellar granule neurons, suggest that trolox, an effective antioxidant, had no protection effect on low K^+ induced-injuries under our experimental conditions.

7. Effects of pretreatment with geldanamycin on glutathione levels in cultured cerebellar granule neurons exposed to low potassium-medium

The exposure of cerebellar granule neurons for 24 hr in serum-free low K^+ medium caused a marked reduction of cellular GSH content to ~ 60% of control while the 24 hr exposure to 0.10 μM GA or 100 μM trolox alone significantly increased cellular GSH content to > 120% of control. However, incubation of cerebellar granule neurons for 24 hr in serum-free low K^+ medium after the pre-exposure to 100 μM trolox for 24 hr did not prevent a decrease in cellular GSH content. Interestingly, pre-exposure to 0.10 μM GA for 24 hr not only prevented low K^+-induced diminution in

cellular GSH content but still boosted the GSH level up to 140% of control (Fig. 31). Therefore, these results suggest that GA possesses a remarked protective effect against K^+ deprivation-induced GSH depletion in cultured cerebellar granule neurons.

8. Effects of pretreatment with geldanamycin on lipid peroxidation in cultured cerebellar granule neurons exposed to low potassium-medium

Switching cultured cerebellar granule neurons from medium containing a high concentration of K^+ (25 mM KCl) to medium containing a low concentration of K^+ (5 mM KCl) for 24 hr significantly increased cellular lipid peroxidation. In contrast, exposure of cultured neurons with 0.10 μM GA or 100 μM trolox alone for 24 hr induced a marked decrease in cellular lipid peroxidation. Pre-exposure to 100 μM trolox or 0.10 μM GA for 24 hr in 25 mM K^+ before switching to 5 mM K^+ for 24 hr significantly prevented an increase in cellular lipid peroxidation (Fig. 32). Therefore, these results suggest that trolox and GA display a protective effect against K^+ deprivation-induced lipid peroxidation in cultured cerebellar granule neurons.

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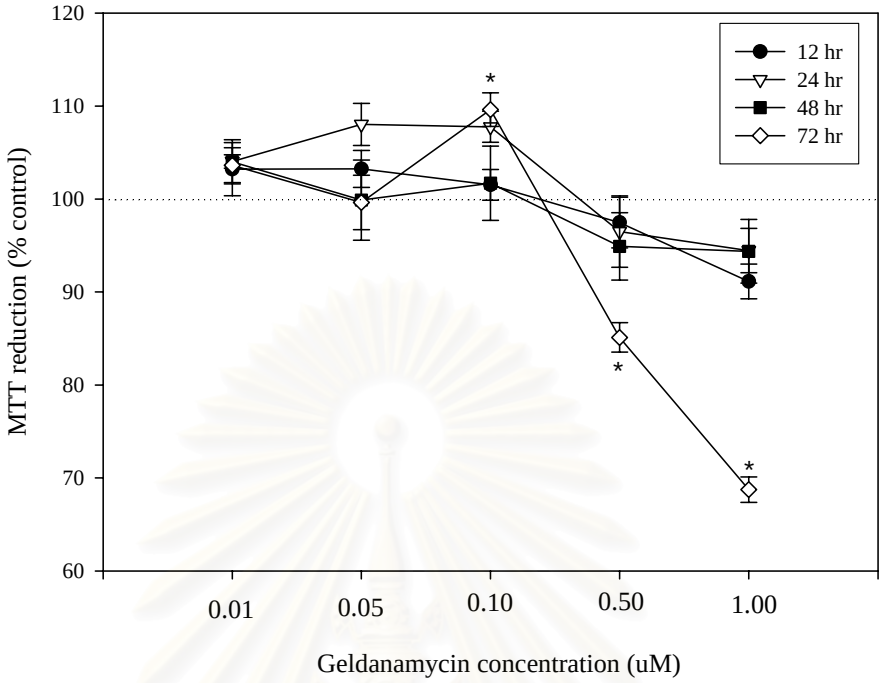

Fig. 5 Effects of geldanamycin on mitochondrial activity in cultured cerebellar granule neurons.

Cerebellar granule neurons were cultured for 8 days and then incubated with various concentrations of GA (0.01-1.0 μM). Neuronal injury was determined after 12, 24, 48, and 72 hr of incubation by examination of mitochondrial dehydrogenase activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

* $p < 0.05$ compared with untreated control (one way ANOVA and Scheff's test)

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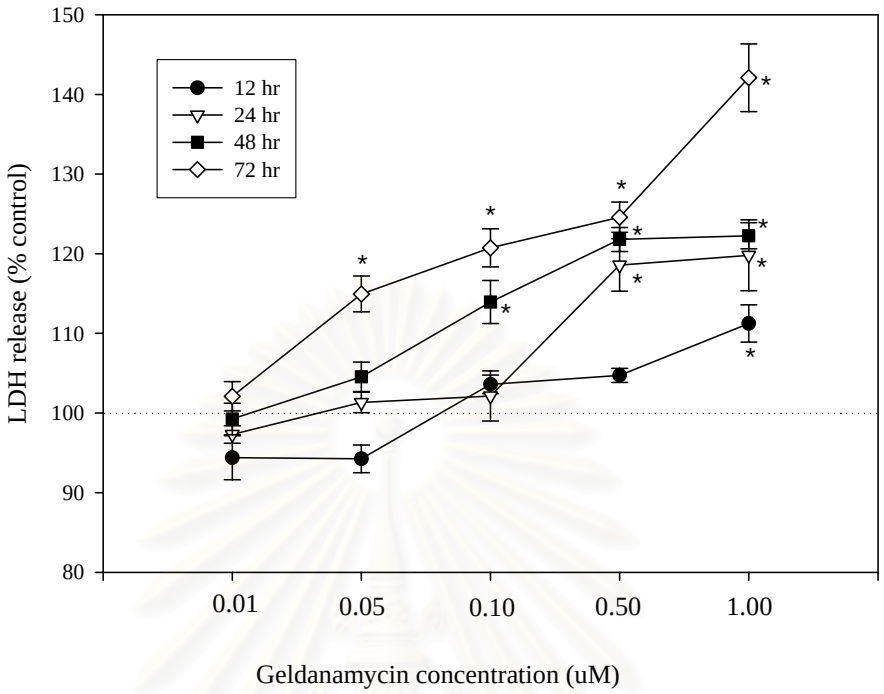

Fig. 6 Effects of geldanamycin on viability of cultured cerebellar granule neurons.

Cerebellar granule neurons were cultured for 8 days and then incubated with various concentrations of GA (0.01-1.0 μM). Neuronal cell death was determined after 12, 24, 48, and 72 hr of incubation by quantifying the released LDH activity. Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ compared with untreated control (one way ANOVA and Scheff's test)

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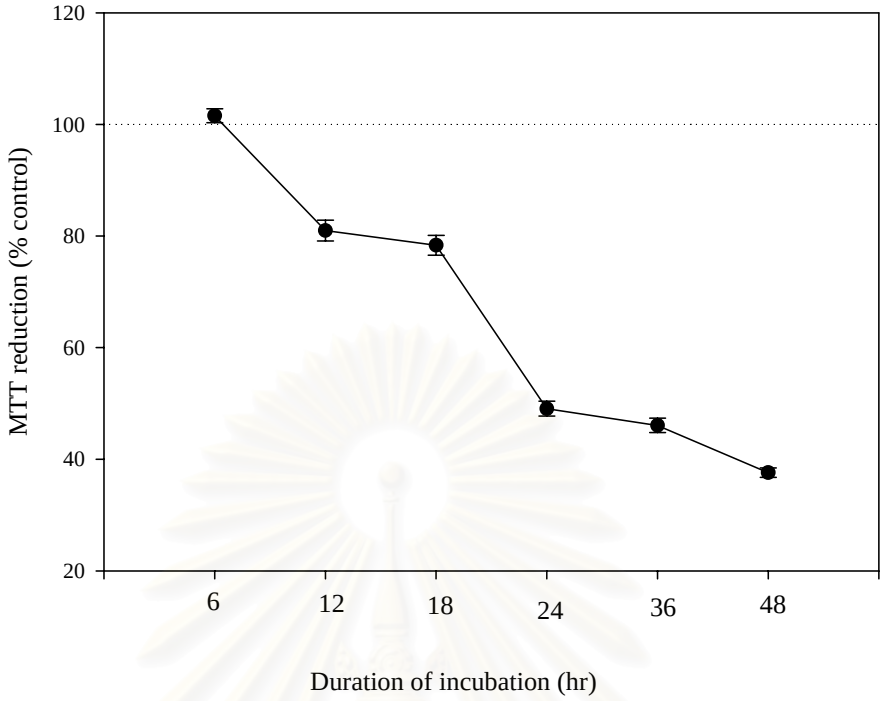

Fig. 7 Time course of granule neuronal injuries induced by low K^+ medium

Cerebellar granule neurons were cultured in medium with 10% fetal bovine serum and 25 mM KCl for 8 days. The medium was replaced with a serum-free DMEM containing 5 mM KCl for 6, 12, 18, 24, 36, and 48 hr before determination of MTT reduction activity. Control cells were switched to a serum-free DMEM containing 25 mM KCl. Results represent the mean ± S.E.M values of twelve samples taken from duplicate experiments.

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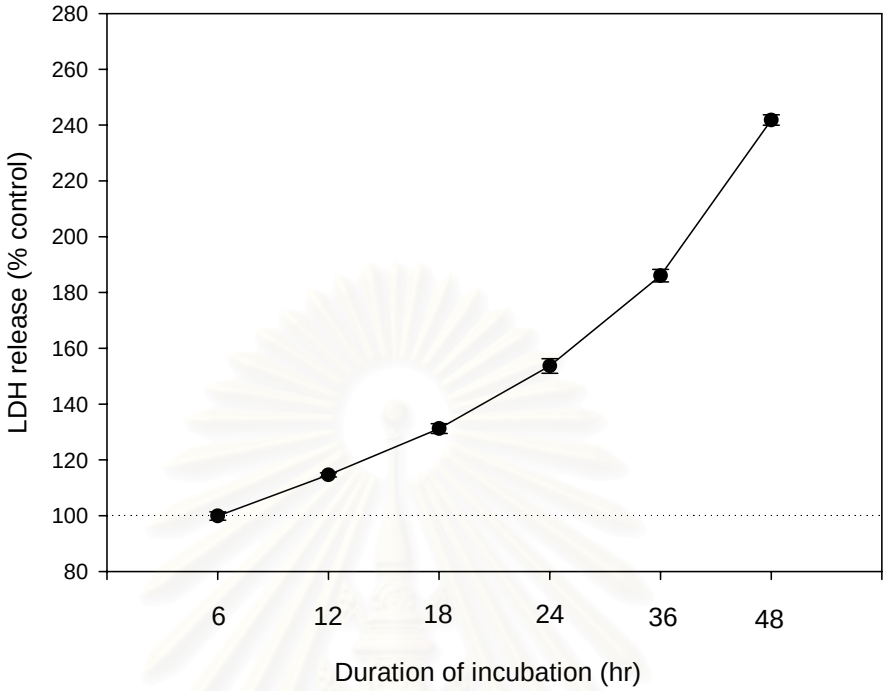

Fig. 8 Time course of granule neuronal cell death induced by low K^+ medium

Eight days after plating, neurons were switched to serum-free DMEM containing low K^+ (5 mM). LDH activity in the culture medium was measured at 6, 12, 18, 24, 36, and 48 hr of incubation. Results represent the mean ± S.E.M values of twelve samples taken from duplicate experiments and are expressed as percentage of total LDH activity in control cultures. In control cultures, neurons were maintained in serum-free with high K^+ (25 mM KCl).

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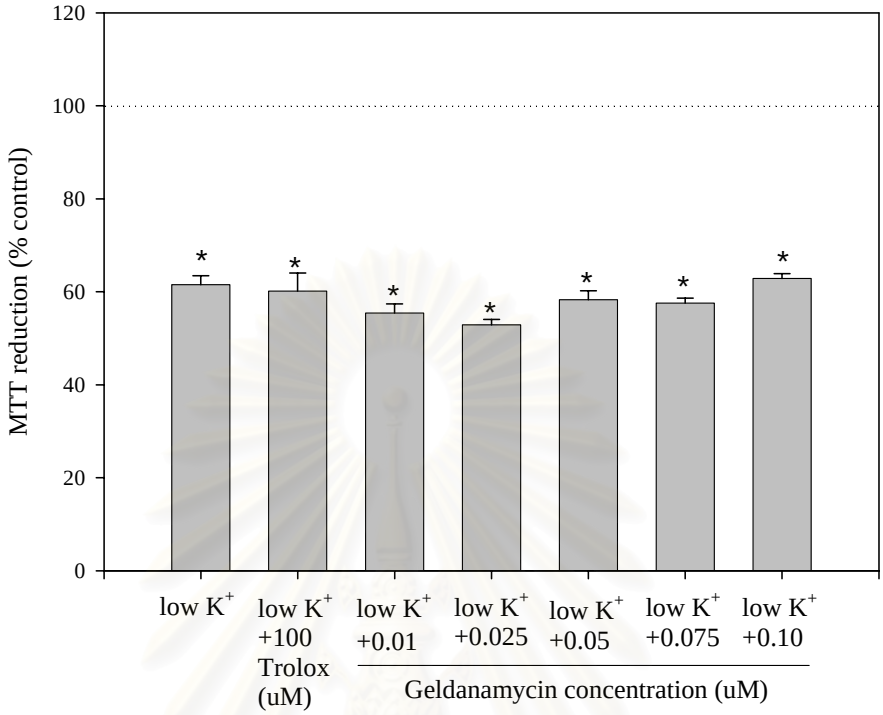

Fig. 9 Effects of coexposure with geldanamycin on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing low K^+ (5 mM KCl) for 24 after which neuronal injury was determined by cellular MTT reduction activity. Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells (one way ANOVA and Scheff's test).

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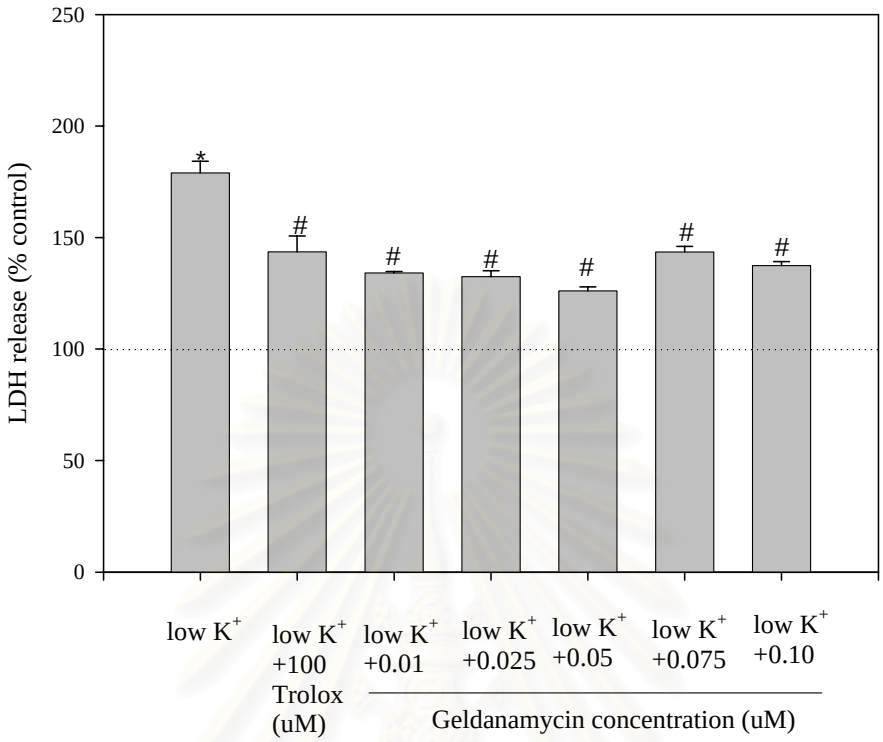

Fig. 10 Effects of coexposure with geldanamycin on low-potassium-induced cell death in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr after which neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells.

*$p < 0.05$ vs untreated control, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

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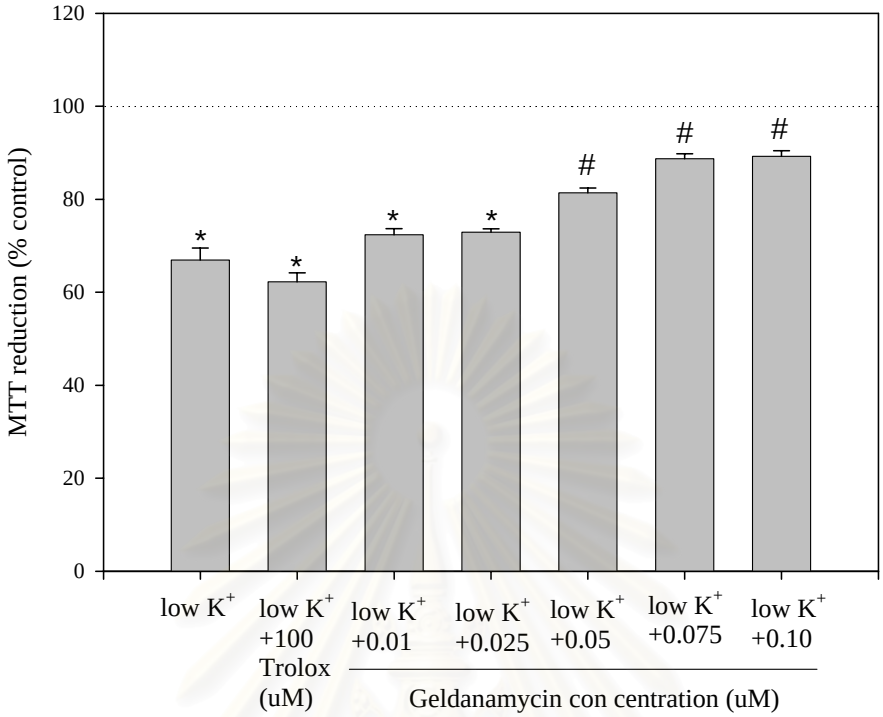

Fig. 11 Effects of 6 hr pre-exposure with geldanamycin on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 6 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

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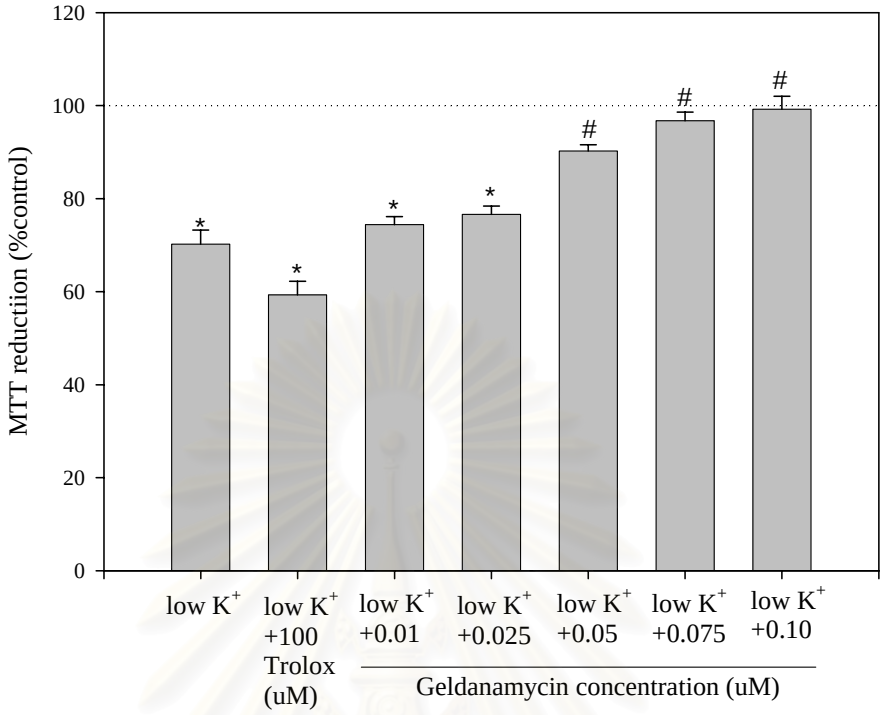

Fig. 12 Effects of 12 hr pre-exposure with geldanamycin on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 12 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

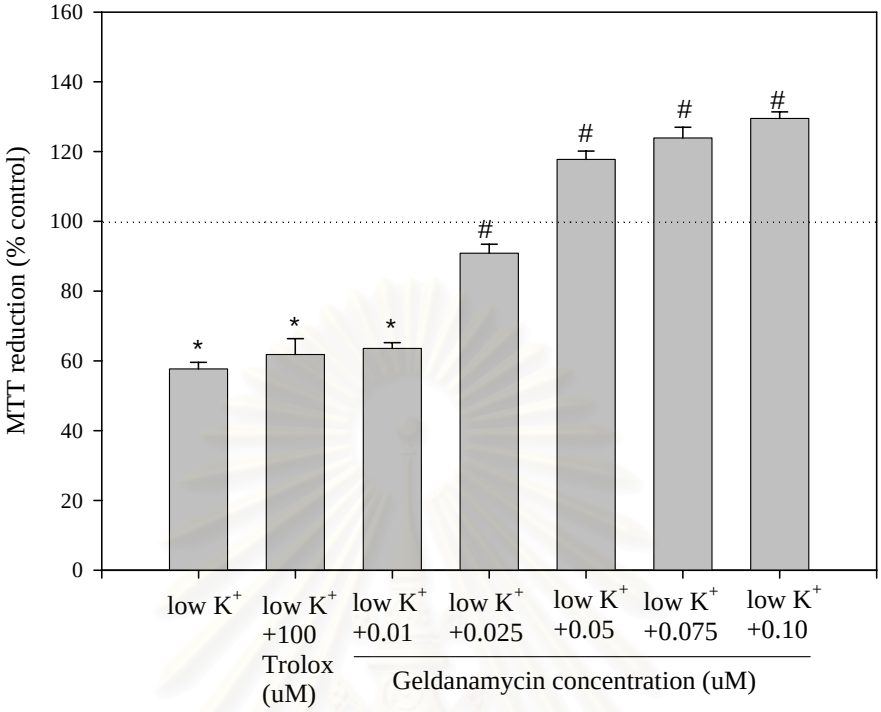

Fig. 13 Effects of 24 hr pre-exposure with geldanamycin on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 24 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

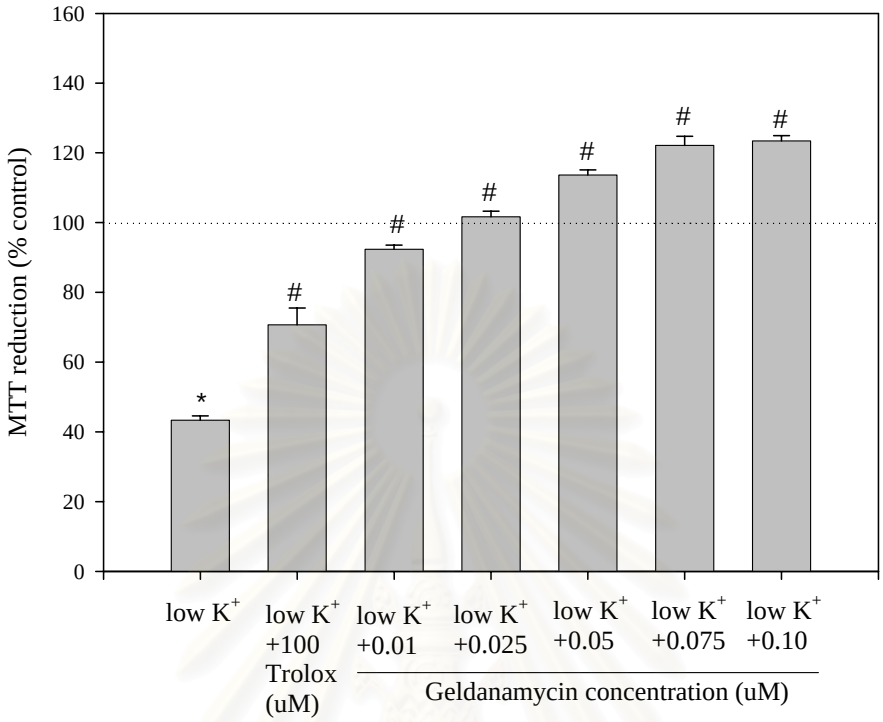

Fig. 14 Effects of 48 hr pre-exposure with geldanamycin on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 48 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

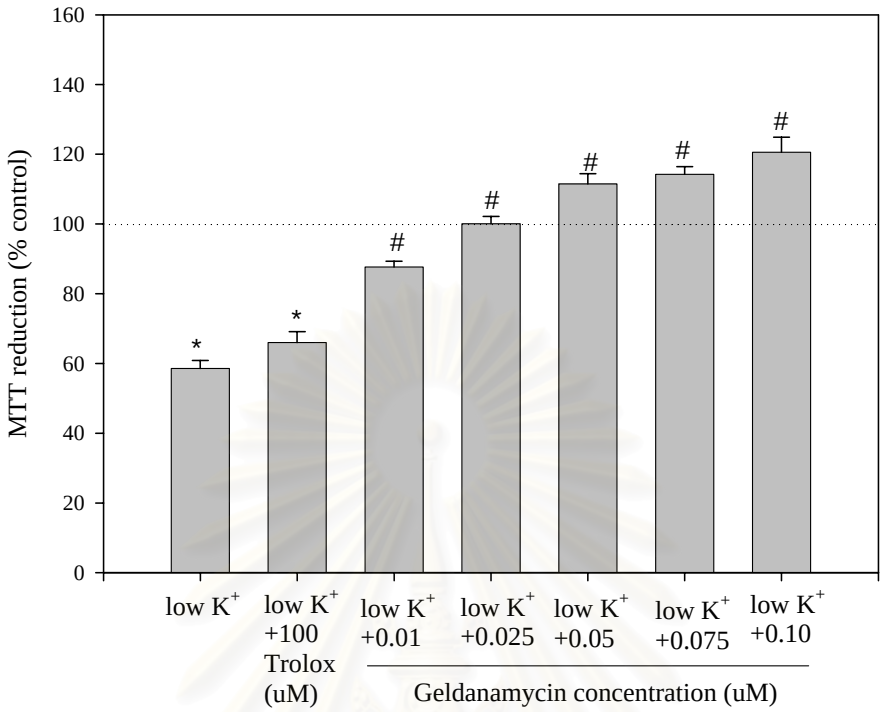

Fig. 15 Effects of 72 hr pre-exposure with geldanamycin on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 72 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

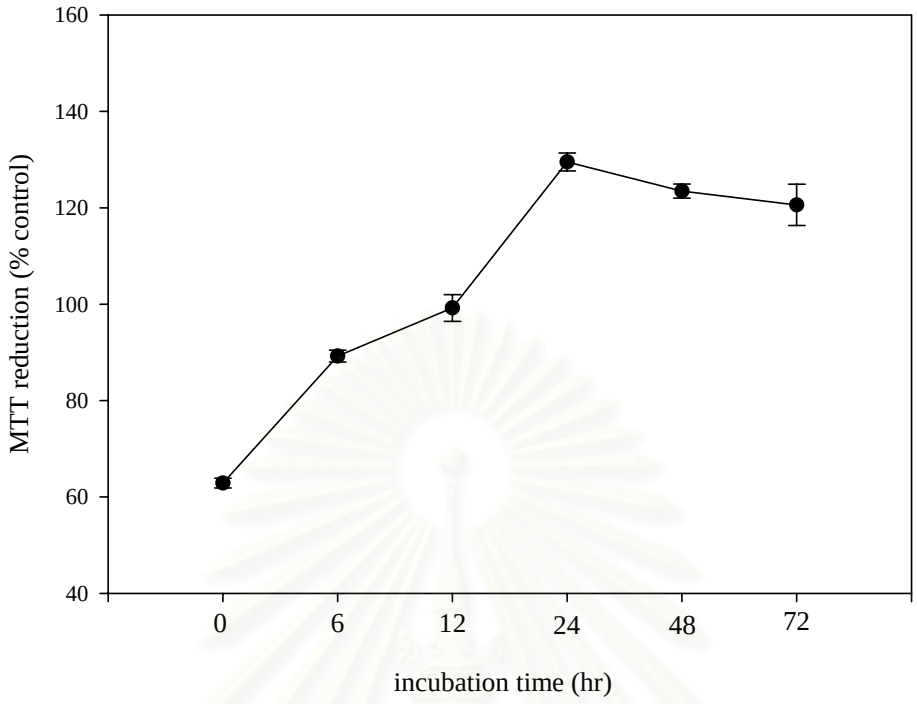

Fig. 16 Effects of pre-exposure with 0.10 μM geldanamycin on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of 0.10 μM GA in serum-free DMEM containing high K^+ (25 mM KCl) for 0, 6, 12, 24, 48, and 72 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

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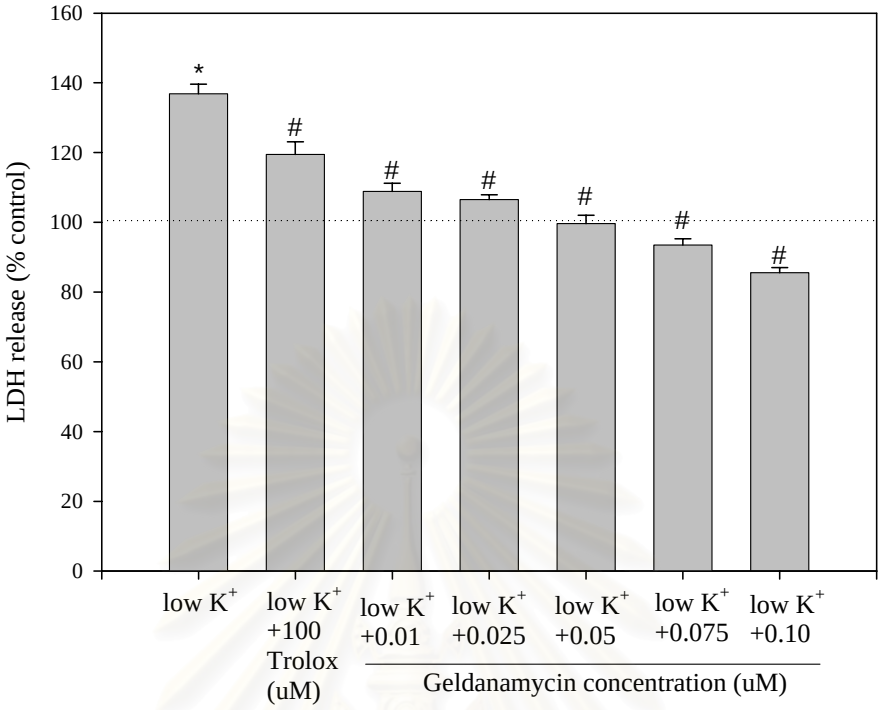

Fig. 17 Effects of 6 hr pre-exposure with geldanamycin on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 6 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells. Data represent mean ± S.E.M values of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

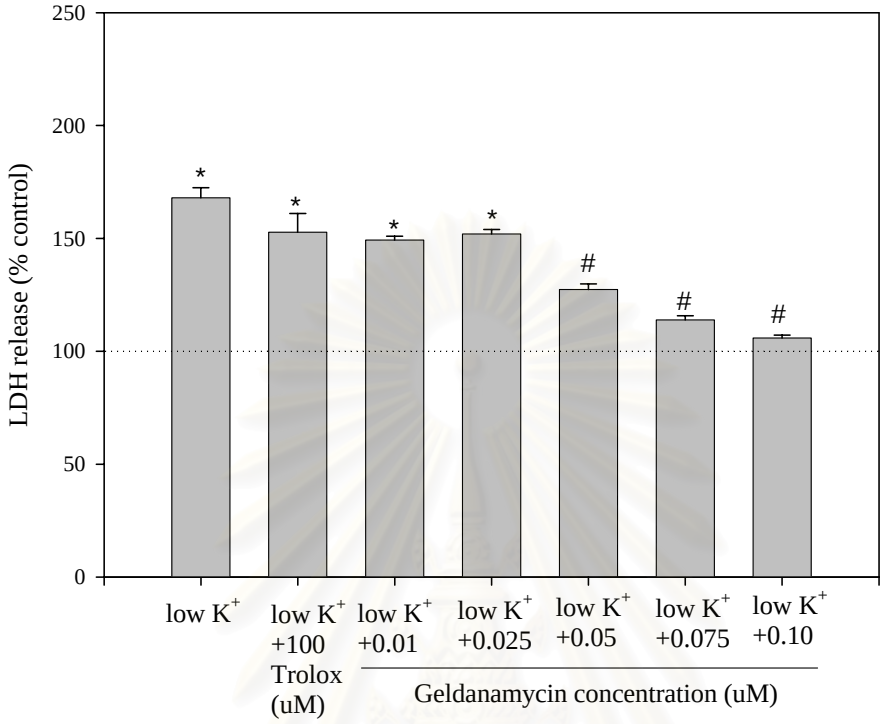

Fig. 18 Effects of 12 hr pre-exposure with geldanamycin on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 12 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells. Data represent mean ± S.E.M values of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

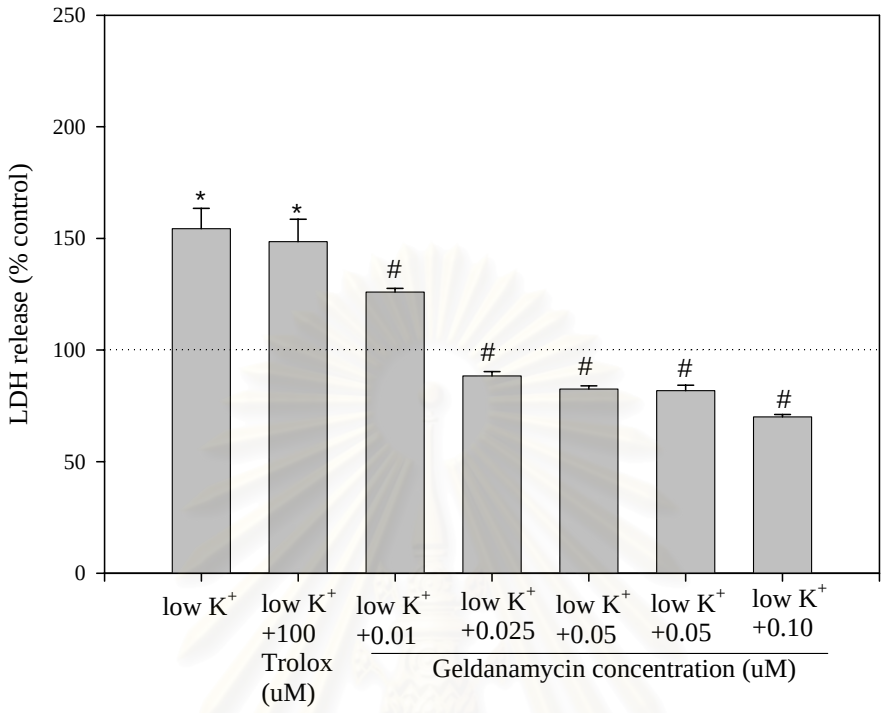

Fig. 19 Effects of 24 hr pre-exposure with geldanamycin on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 24 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells. Data represent mean ± S.E.M values of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

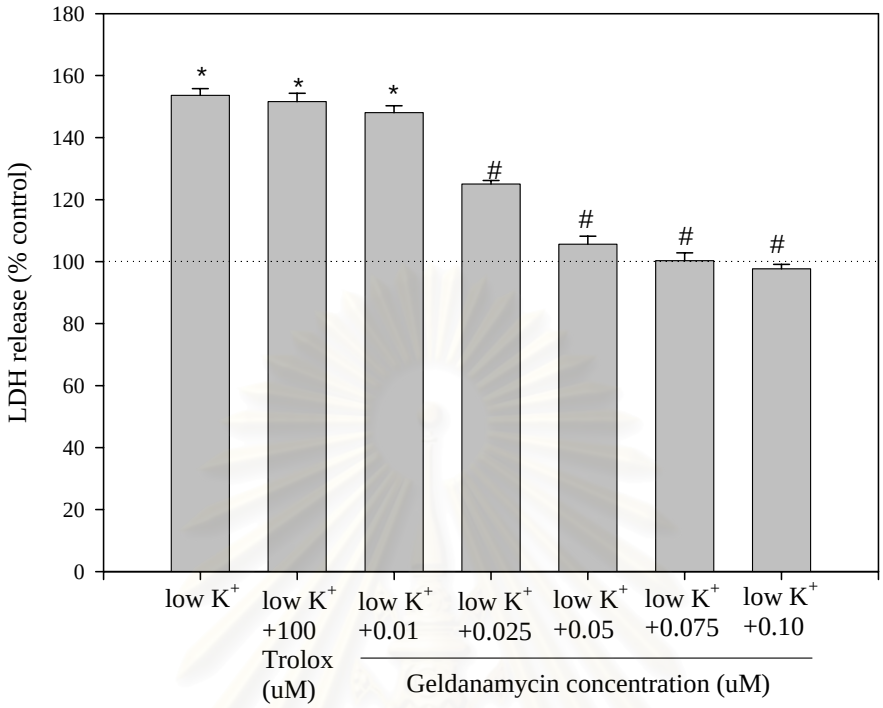

Fig. 20 Effects of 48 hr pre-exposure with geldanamycin on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 48 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells. Data represent mean ± S.E.M values of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

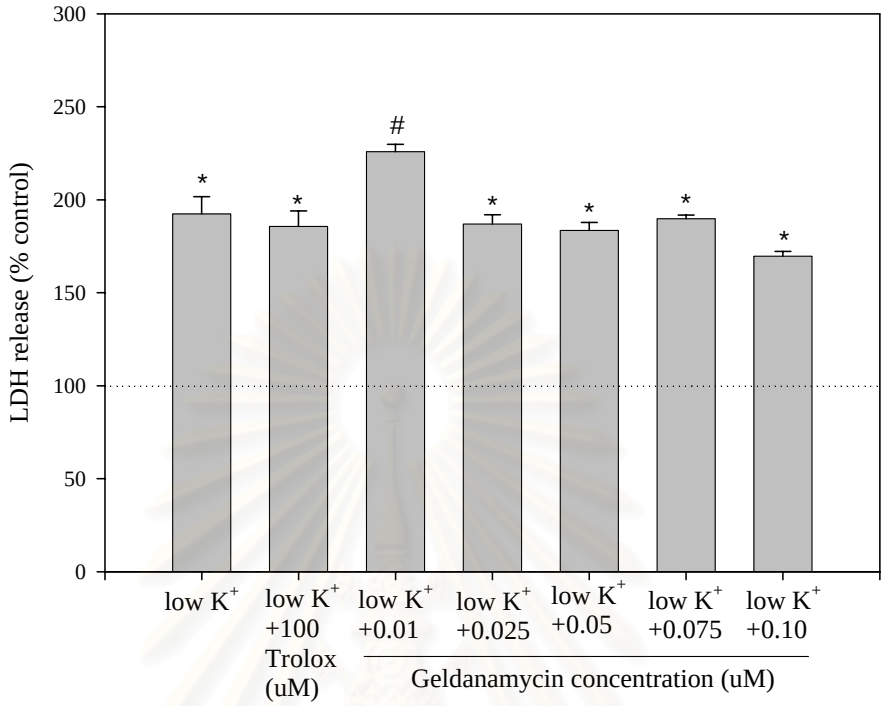

Fig. 21 Effects of 72 hr pre-exposure with geldanamycin on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of GA (0.01-0.10 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 72 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells. Data represent mean ± S.E.M values of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

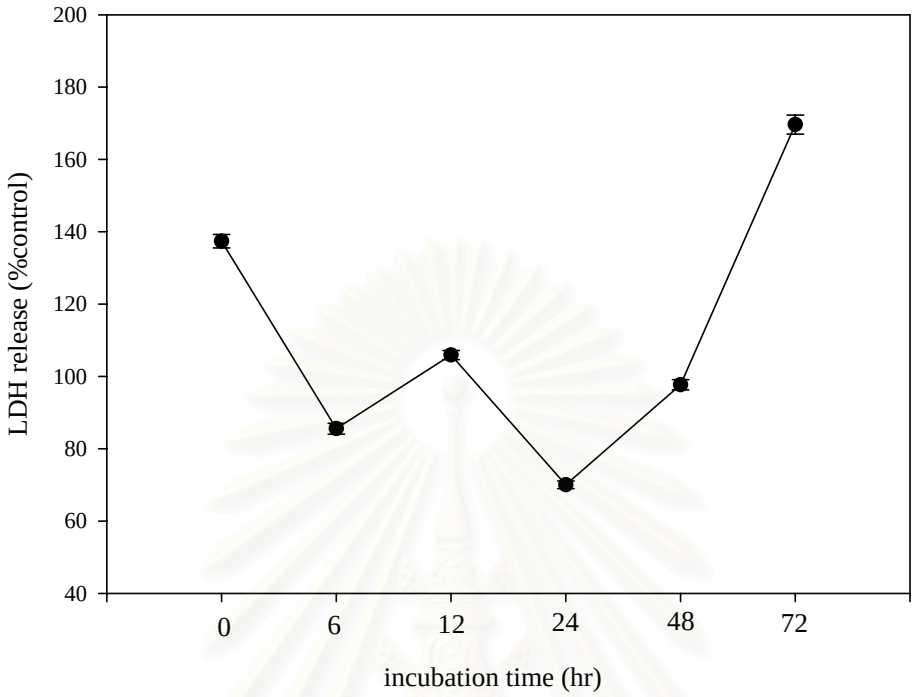

Fig. 22 Effects of pre-exposure with 0.10 μM geldanamycin on low-potassium-induced cell death in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of 0.10 μM GA in serum-free DMEM containing high K^+ (25 mM KCl) for 0, 6, 12, 24, 48, and 72 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells. Data represent mean ± S.E.M values of eight samples from duplicate experiments.

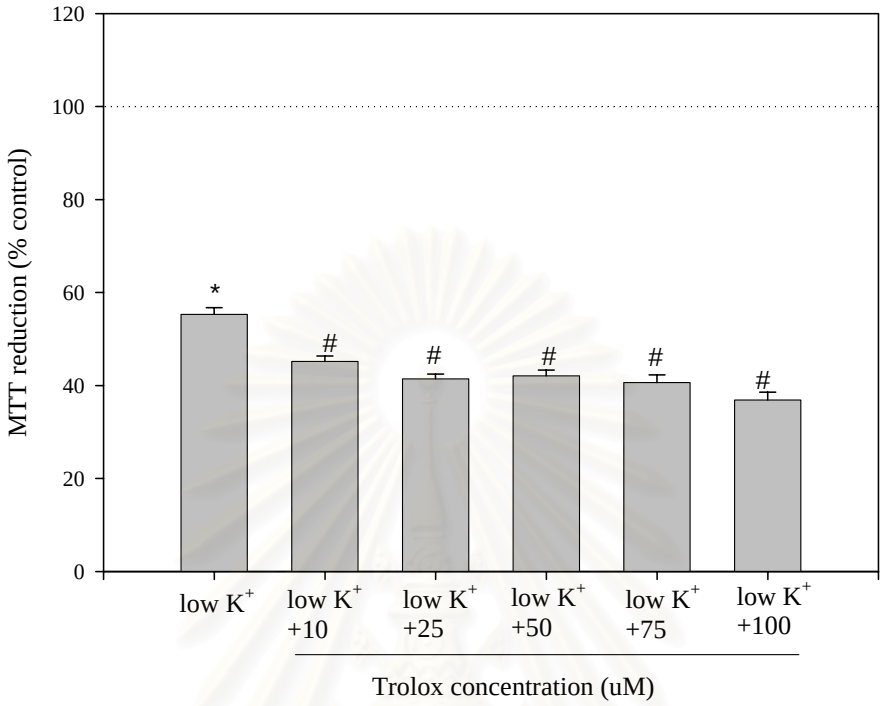

Fig. 23 Effects of coexposure with trolox on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

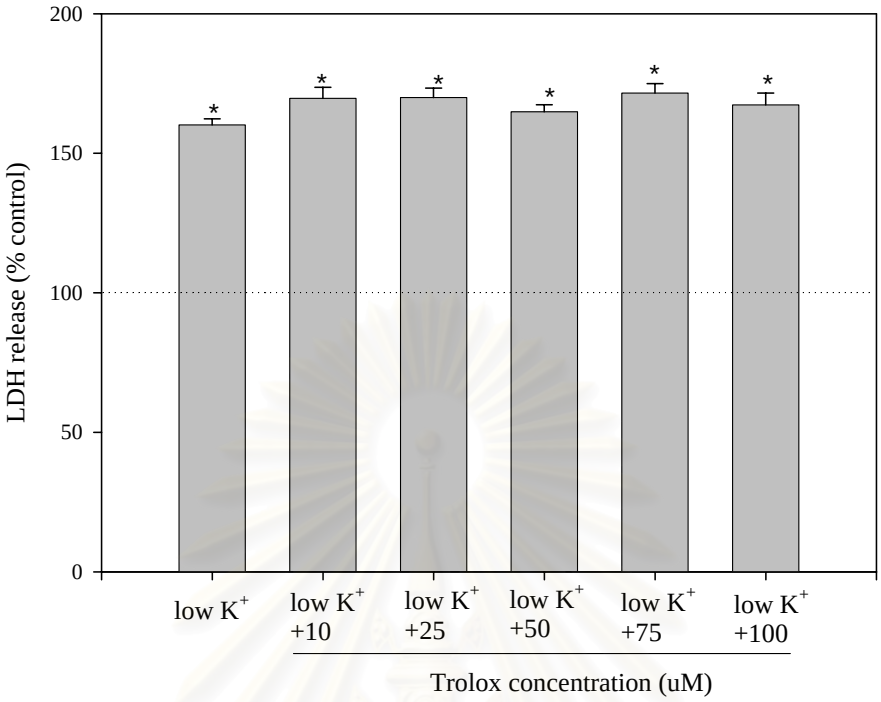

Fig. 24 Effects of coexposure with trolox on low-potassium-induced injuries in cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as a percentage of values from 25 mM K^+-untreated control cells. Data represent mean ± S.E.M values of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

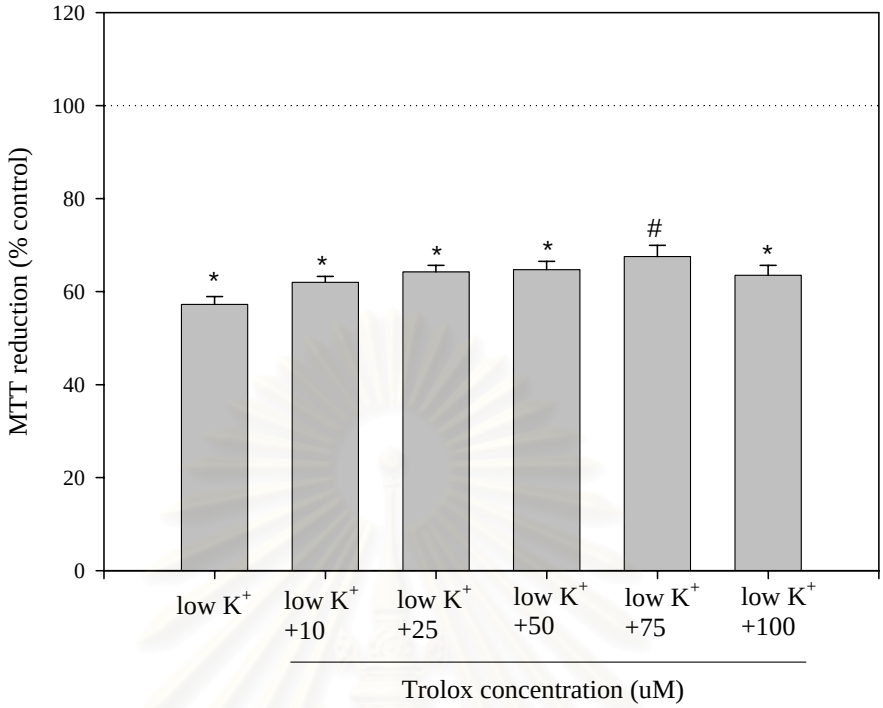

Fig. 25 Effects of 24 hr pre-exposure with trolox on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 24 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

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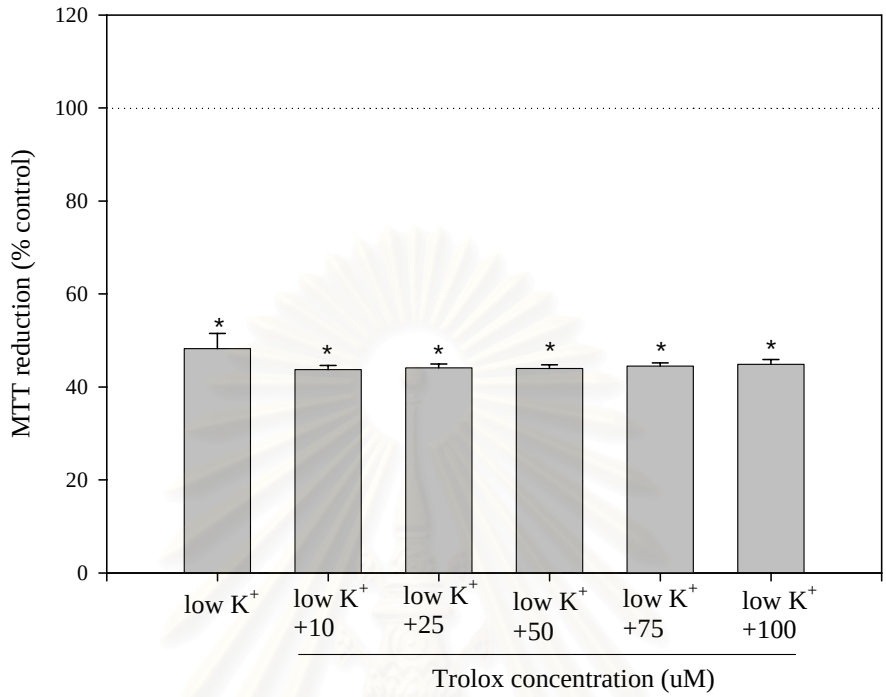

Fig. 26 Effects of 48 hr pre-exposure with trolox on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 48 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

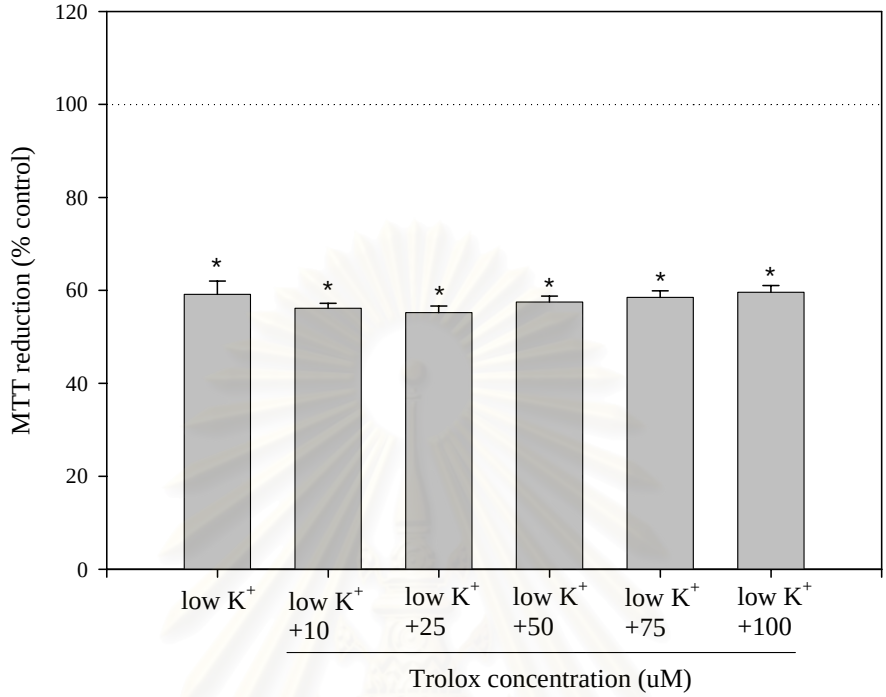

Fig. 27 Effects of 72 hr pre-exposure with trolox on low-potassium-induced injuries in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 72 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Cell injury was determined by mitochondrial metabolic activity (MTT reduction). Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

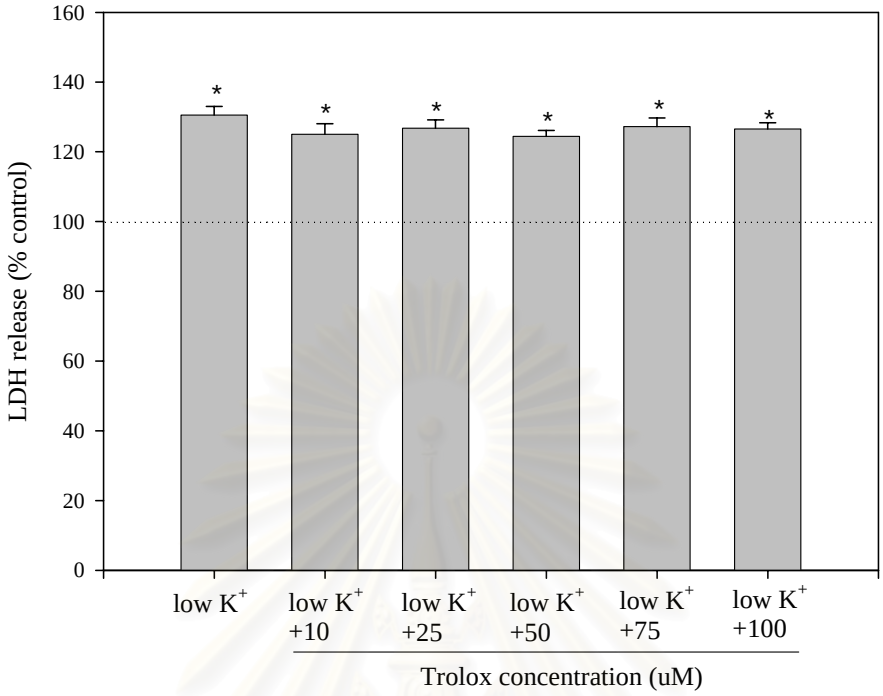

Fig. 28 Effects of 24 hr pre-exposure with trolox on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 24 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

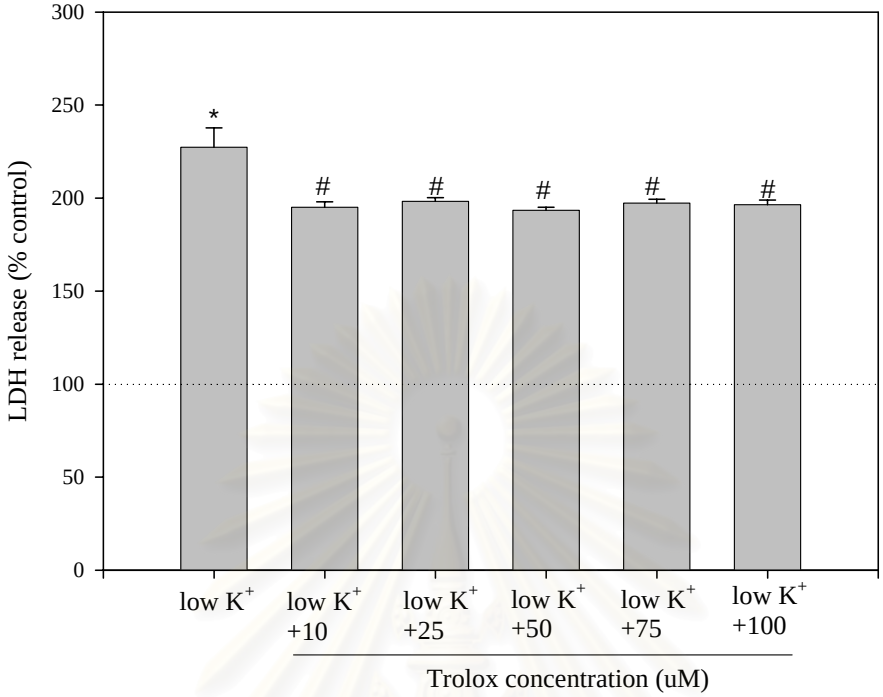

Fig. 29 Effects of 48 hr pre-exposure with trolox on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 48 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

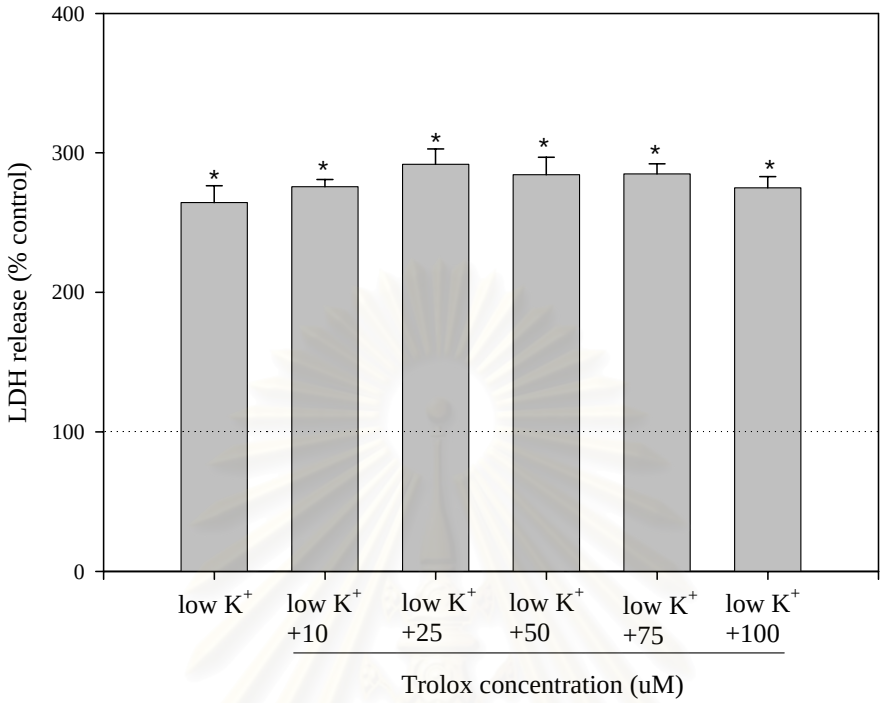

Fig. 30 Effects of 72 hr pre-exposure with trolox on low-potassium-induced cell death in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of trolox (10-100 μM) in serum-free DMEM containing high K^+ (25 mM KCl) for 72 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr. Neuronal cell death was determined by quantifying the released LDH. Results are expressed as percentage of values from 25 mM K^+-untreated control cells. Data are presented as mean ± S.E.M of eight samples from duplicate experiments.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

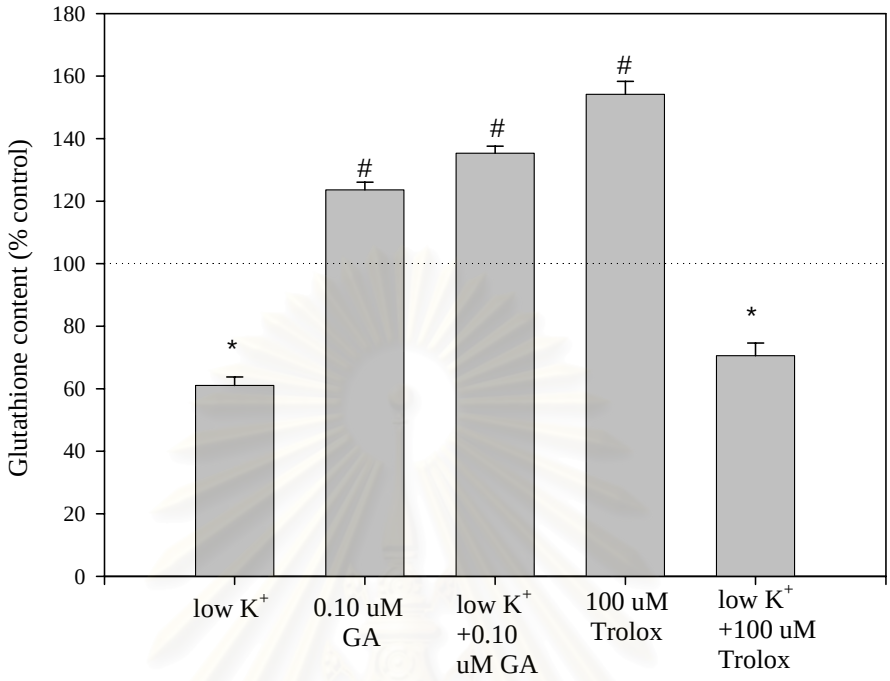

Fig. 31 Effects of pre-exposure with geldanamycin on low-potassium-induced glutathione diminution in cultured cerebellar granule neurons

Cultured cerebellar granule neurons were exposed to various concentrations of 0.10 μM GA in serum-free DMEM containing high K^+ (25 mM KCl) for 24 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr after which cellular GSH contents were measured. Data represent mean ± S.E.M values of eight samples.

*$p < 0.05$ vs untreated control cells, $^{\#}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

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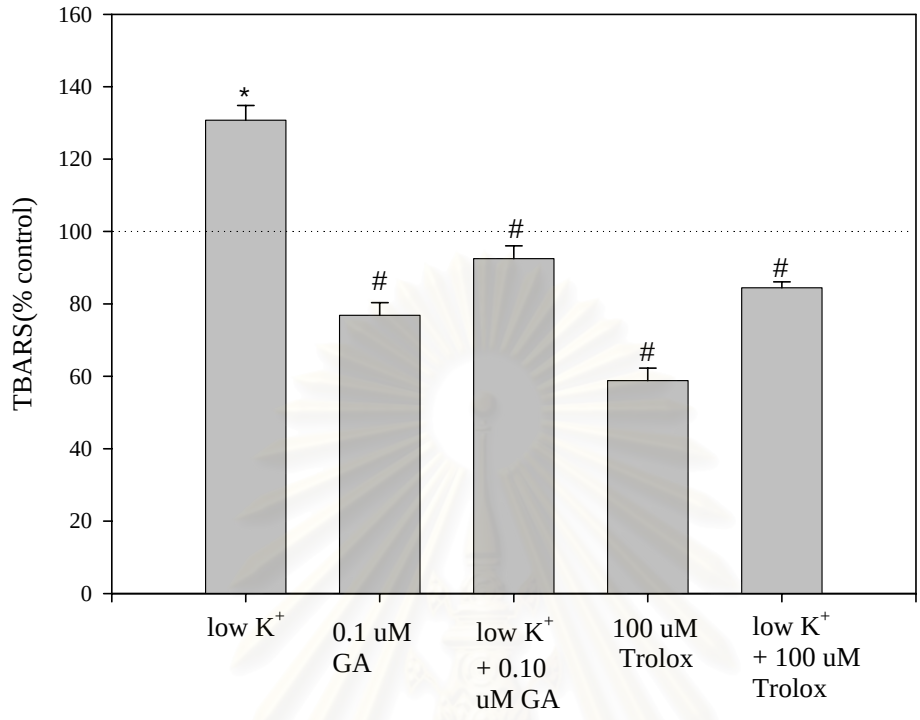

Fig. 32 Effects of pre-exposure with geldanamycin on low-potassium-induced lipid peroxidation in cultured cerebellar granule neurons.

Cultured cerebellar granule neurons were exposed to various concentrations of 0.10 μM GA in serum-free DMEM containing high K^+ (25 mM KCl) for 24 hr and then switched to serum-free DMEM containing low K^+ (5 mM KCl) for 24 hr after which lipid peroxidation was measured by TBARS assay. Data represent mean ± S.E.M values of eight samples.

*$p < 0.05$ vs untreated control cells, $^{**}p < 0.05$ vs low K^+ (5 mM KCl) (one way ANOVA and Scheff's test).

CHAPTER V

DISCUSSION AND CONCLUSION

GA, an inhibitor of the HSP90 chaperone, has been reported to have several cellular effects, such as inhibition of v-src activity or destabilization of Raf-1 among others. Lopez-Maderuelo et al (2001) showed that GA treatment induces different phenotypes in different cell lines. In PC12 cells, it triggers apoptosis, whereas in the murine neuroblastoma N2A, it induces differentiation with neurite outgrowth. Mitogen-activated protein kinase activities ERK and JNK are activated differently according to cell type: in PC12 cells JNK is activated, and its inhibition abolishes apoptosis, but not ERK; in N2A cells, both ERK and JNK are activated, but with peak activities at different times.

Regarding to cell death, GA was reported to protect NRK-52E cells from okadaic acid-induced apoptosis, abrogated the overall okadaic acid-induced kinase activation, and specifically inhibited activation of p38 kinase by okadaic acid (Davis and Carbott, 1999). These results imply that decreased p38 activity and its cytosolic translocation together with cellular resistance to cytoskeletal disorganization may play a significant role in resistance to phosphorylation-dependent apoptosis. Recently, Sano (2001) found that GA at low doses (about 2 nM) appeared to be neurotrophic on DRG neurons in the presence or absence of neurotrophins, but higher doses of geldanamycin (> 5 nM) had severe cytotoxic effects on neurons. GA at low doses was also found to be neuroprotective against anti-cancer drugs. It was suggested that the neurotrophic effects of GA may be due to the association of GA with a member of the HSP90 family. Decreases in the levels of some specific protein(s) that requires HSP90 for its activity may interfere with various signal transduction pathways involving in cell injury and cell death.

As motivated by multimodal effects of GA on cell proliferation and differentiation, this study was designed to investigate a potential cytoprotective effect of GA against K^+ deprivation-induced cell injuries in primary cultured rat cerebellar granule cells. It was initially hypothesized that GA might possess neuroprotective

effects, at least, *in vitro*. Apparently, experimental results from this study did support that speculation. It is notable that GA might exert remarkable neuroprotective effect if it had exposed to the neurons for a certain period prior to the insulting agent.

Effects of geldanamycin on cultured rat cerebellar granule cells and on low K^+–induced neuronal injuries in these cells were investigated by using cell metabolic activity and viabillity, content of glutathione and lipid peroxidation, as the measuring endpoints. Eight-day cultured cerebellar granule cells were used in all experiments. Exposure of cultured cerebellar granule neurons for 12-72 hr to geldanamycin at different concentrations (0.01-1.0 μM) showed concentration- and exposure time-dependent neuroprotective effects. At low concentrations of GA with short incubation periods, GA showed a little effect on neuronal metabolic activity. However, after 24-72 hr pre-exposure to GA at concentrations higher than 0.05 μM, GA had a marked effect on neuronal metabolic activity. With 24 hr pre-exposure to GA at concentrations higher than 0.025 μM, cell death caused by low K^+ was significantly prevented. Apparently, the maximal magnitude of protection may be obtained from 24 hr pre-exposure to 0.10 μM GA.

Switching cultured neurons from medium containing 25 mM K^+ to medium containing 5 mM K^+ caused granule cell death mostly by apoptosis. Simultaneous exposure with GA and low K^+ for 24 h did not protect cell death. However, pretreatment of 0.1 μM GA in high K^+ condition for 24 h before subjecting to low K^+ condition effectively prevented granule cell death. Experimental results indicated that the cell survival rate was only approximately 50% after exposure to low K^+ for 24 h. In contrast, the cell survival rate was markedly enhanced up to that of cells maintained in high K^+ when pretreating with GA for a certain period of time. Furthermore, GSH assay revealed that total GSH content was reduced by nearly 40% after switching cultured neurons to low K^+ for 24 h. Pretreatment with 0.1 μM GA for 24 h effectively inhibited low K^+-induced GSH diminution. Moreover, the same pretreatment with GA also prevented an increase in cellular lipid peroxidation after 24-h exposure to low K^+. These findings suggest that GA pretreatment may prevent granule cell death by induction of proteins that regulate GSH metabolism and lipid peroxidation. In contrast, trolox, a soluble antioxidant compound which is pharmacological standard of this study for comparison, had no protection effect on low K^+ induced-injuries. Unchern et

al.(1997) found that trolox had no protection on low K^+ induced-injuries in cultured rat cerebellar granule neurons.

The highly conserved heat shock proteins (HSPs) accumulate in cells exposed to heat and a variety of other stressful stimuli including low K^+ condition. HSPs, which function mainly as molecular chaperones, allow cells to adapt to gradual changes in their environment and to survive in otherwise lethal conditions. The events of cell stress and cell death are linked and HSPs induced in response to stress appear to function at key regulatory points in the control of apoptosis. HSPs include antiapoptotic and proapoptotic proteins that interact with a variety of cellular proteins. Their expression level can determine the fate of the cell in response to a death stimulus, and apoptosis-inhibitory HSPs, in particular HSP27 and HSP70, may participate in carcinogenesis (Garrido et al., 2001).

GA, a 90-kDa heat shock protein (HSP90) specific inhibitor, is a well-known potential anticancer agent. Administration of GA to cells can rapidly and selectively inhibit the activities to HSP90 and of its substrates, thereby destabilizing client proteins. Its ability to simultaneously stimulate depletion of multiple oncogenic proteins suggests that GA may contribute to cancer therapy (Huang et al., 2002). The disruption of HSP 90 function that results from GA binding is likely to be responsible for many indirect effects of GA, including its ability to inhibit certain tyrosine kinases (whitesell et al., 1994) and induce HSP70 expression (Conde et al., 1997).

In addition to inducing the activation of heat shock factor 1 (HSF1), GA increases the synthesis and cellular levels of heat shock proteins, particularly 70-kDa heat shock protein (HSP70). Conde et al. (1997) demonstrated that exposure of rat neonatal cardiomyocytes and H9c2 cells to GA induces heat shock proteins, thus providing protection against ischemic stress. These results suggest that GA may offer a pharmacological means of increasing the level of heat shock proteins in cardiac tissue and, in doing so, protecting the heart against ischemic/ reperfusion injury.

HSP 70 has been found to play a role in protection against apoptosis in several different cell types (Wei et al., 1995, Sato et al., 1996, Mosser et al., 1997). In addition, HSP70 appears to be responsible for the neuroprotective effects of heat

shock (Sato et al., 1996). Xiao et al. (1999) found that 0.1 μg/ml GA effectively induced HSP 70 expression in HT22 cells.

Huang et al. (2002) found that GA inhibits trichostatin A-induced cell death and histone H4 hyperacetylation in COS-7 cells. The accumulated 70-kDa heat shock protein (HSP70) markedly increased up to 2 to 3 folds at 8 h in GA-treated cells, and the maximum amount up to 5 to 7 folds at 20 h. Conversely, HSP90 did not markedly increase in all treatments.

As an alternative explanation, based on a number of recent studies, it is possible that apoptotic cell death induced by low K^+ condition might be prevented by GA-induced increment of heat shock proteins, particularly HSP70.

The protective role of HSPs in cell death remains contentious. First, HSPs do not have the same protective ability in all apoptosis models. Second, some studies reveal no protective effects of HSPs in apoptosis at all, although these molecules are induced during apoptosis. Third, in some models, HSPs have been considered as apoptosis enhancers: overexpression of HSP70 enhances T-cell receptor/CD3- and Fas/Apo-1/ CD95-mediated apoptosis in Jurkat T cells, and overexpression of HSP90 increases the tumor necrosis factor (TNF)-α- -and cycloheximide-induced apoptosis of the human U-937 cell line. Such conflicting data on HSP functions in apoptosis have been tentatively explained by the differential mechanisms used by distinct cells to respond to different apoptosis-inducing stimuli (Conde et al., 1997; Huang et al., 2002).

Recent findings suggest that unfolded or misfolded proteins participate in the pathology of several neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease. Usually, several stress proteins and glial cells act as intracellular molecular chaperones and show chaperoning neuronal function, respectively. In the brains of patients with neurodegenerative disorders, however, stress proteins are expressed and frequently associated with protein aggregates, and glial cells are activated around degenerative regions. In addition, several stress proteins and glial cells may also regulate neuronal cell death and loss. Therefore, some types of stress proteins and glial cells are considered to be neuroprotective targets (Kitamura and Nomura, 2003; Pavlik et al, 2003). Therefore, it is conceivable to search for potential

substances which modulate stress protein and glial cell functions. This approach through which GA is a potential candidate may be a new therapeutic strategy to treat neurodegeneration (Parcellier et al, 2003; Sreedhar and Csermely, 2004).

In conclusion, experimental results in this study suggest that GA might possess a remarkable neuroprotective effect against K^+ deprivation-induced neuronal injury and death. GA pretreatment may prevent neuronal cell damages by induction of proteins that regulate GSH metabolism and cellular lipid peroxidation. In addition, GA may counteract with low K^+-induced cell death because GA increases induction of HSPs, a group of potential factors to protect cells from damage.

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1. Effects of geldanamycin on MTT reduction in cultured cerebellar granule neurons

Table 1.1 : 12 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	109.90	107.26	107.26	109.29	96.90
Group 2	100	115.99	105.23	105.23	102.79	97.71
Group 3	100	110.51	102.79	102.79	98.73	93.85
Group 4	100	100.15	106.45	106.45	102.99	92.64
Group 5	100	101.49	99.03	99.03	98.68	92.01
Group 6	100	98.68	100.44	100.44	90.61	86.39
Group 7	100	92.01	96.22	96.22	88.49	83.23
Group 8	100	96.93	94.82	94.82	88.15	86.39
Mean	100	103.21	101.53	101.53	97.47	91.14
S.E.M	0	2.86	1.99	1.65	2.73	1.86

Table 1.2 : 24 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	107.00	113.56	114.61	110.98	104.24
Group 2	100	104.24	113.92	111.49	110.97	104.06
Group 3	100	102.43	107.10	102.17	91.41	93.92
Group 4	100	104.33	107.97	107.97	104.58	93.07
Group 5	100	106.94	97.69	108.65	94.43	95.97
Group 6	100	103.34	116.53	103.17	86.72	87.96
Group 7	100	101.79	105.23	102.49	88.39	86.09
Group 8	100	102.24	102.23	111.61	84.49	90.32
Mean	100	104.04	108.03	107.77	96.49	94.46
S.E.M	0	0.72	2.26	1.67	3.83	2.39

Table 1.3 : 48 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	101.53	92.79	109.49	104.69	91.27
Group 2	100	97.76	90.95	92.54	89.08	75.17
Group 3	100	101.02	118.17	116.39	105.16	101.12
Group 4	100	108.97	115.52	113.33	106.44	103.97
Group 5	100	96.10	96.47	96.47	81.15	85.98
Group 6	100	108.91	93.78	106.78	84.78	98.05
Group 7	100	116.03	106.78	93.78	87.89	100.82
Group 8	100	101.72	84.62	84.82	100.05	98.59
Mean	100	104.01	99.89	101.70	94.91	94.37
S.E.M	0	2.37	4.31	4.00	3.62	3.43

Table 1.4 : 72 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	105.15	104.07	113.28	80.94	67.03
Group 2	100	107.32	111.65	113.09	86.54	64.14
Group 3	100	98.64	100.63	109.12	82.75	74.25
Group 4	100	96.66	84.01	111.83	82.93	72.81
Group 5	100	103.04	94.39	112.79	87.58	72.13
Group 6	100	100.28	105.24	99.36	80.04	66.05
Group 7	100	113.34	100.46	104.51	93.84	64.77
Group 8	100	104.69	96.59	112.97	86.29	68.74
Mean	100	103.64	99.63	109.62	85.11	68.74
S.E.M	0	1.87	2.92	1.81	1.58	1.37

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2. Effects of geldanamycin on LDH release in cultured cerebellar granule neurons

Table 2.1 : 12 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	94.80	90.79	107.89	103.01	113.34
Group 2	100	102.42	85.69	106.15	105.47	121.10
Group 3	100	85.93	91.07	101.69	101.56	108.80
Group 4	100	83.18	95.87	103.95	107.39	110.59
Group 5	100	98.73	98.28	100.02	105.07	102.61
Group 6	100	100.87	100.82	100.98	103.65	111.05
Group 7	100	101.91	97.66	100.33	102.60	103.37
Group 8	100	87.29	93.89	107.81	109.02	119.04
Mean	100	94.39	94.26	103.60	104.72	111.24
S.E.M	0	2.77	1.74	1.17	0.89	2.33

Table 2.2 : 24 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	98.66	105.47	117.25	118.04	138.41
Group 2	100	93.49	105.66	114.49	120.88	128.16
Group 3	100	101.32	96.60	99.70	117.98	125.85
Group 4	100	97.33	99.41	98.95	108.83	120.07
Group 5	100	93.33	104.47	91.81	104.72	106.98
Group 6	100	101.32	97.88	100.27	127.93	103.77
Group 7	100	97.59	98.59	98.52	133.48	128.16
Group 8	100	95.45	102.57	96.11	116.79	107.00
Mean	100	97.31	101.33	102.14	118.58	119.80
S.E.M	0	1.10	1.29	3.15	3.29	4.45

Table 2.3 : 48 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	102.25	98.89	103.32	119.82	120.39
Group 2	100	93.41	96.17	102.33	120.87	117.93
Group 3	100	96.71	107.39	114.91	129.96	124.99
Group 4	100	91.96	104.67	121.36	120.80	122.30
Group 5	100	96.99	103.07	113.13	123.39	114.05
Group 6	100	101.85	108.26	123.16	114.61	125.03
Group 7	100	109.10	112.45	116.69	122.23	126.90
Group 8	100	101.74	105.40	116.58	122.71	127.28
Mean	100	99.25	104.54	113.94	121.79	122.26
S.E.M	0	1.97	1.84	2.69	1.51	1.64

Table 2.4 : 72 hr of incubation

	Control	0.01 μM	0.05 μM	0.1 μM	0.5 μM	1 μM
Group 1	100	97.59	117.67	113.18	117.82	145.36
Group 2	100	110.88	115.69	113.73	129.64	144.17
Group 3	100	106.69	121.86	128.16	129.81	149.03
Group 4	100	105.30	123.95	122.74	125.57	148.68
Group 5	100	101.95	113.80	122.82	122.20	143.63
Group 6	100	99.86	108.75	131.32	118.17	157.89
Group 7	100	95.35	113.14	119.33	121.89	123.75
Group 8	100	99.21	104.76	114.54	131.56	124.28
Mean	100	102.10	114.95	120.73	124.58	142.09
S.E.M	0	1.83	2.25	2.39	1.90	4.25

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3. Time course of low K^+-induced injuries in cultured cerebellar granule neurons

Table 3.1 : MTT reduction assay

	Control	6 hr	12 hr	18 hr	24 hr	36 hr	48 hr
Group 1	100	103.06	83.59	79.82	53.26	72.38	34.36
Group 2	100	99.19	77.37	89.48	46.47	51.17	36.23
Group 3	100	97.34	71.48	76.80	43.59	41.92	40.29
Group 4	100	106.81	70.14	75.29	52.69	50.29	39.88
Group 5	100	105.76	94.04	88.38	45.59	47.23	33.11
Group 6	100	103.04	77.65	75.00	47.59	41.01	40.37
Group 7	100	97.18	82.38	73.62	47.86	47.19	35.28
Group 8	100	95.99	85.99	68.42	41.09	40.23	41.51
Group 9	100	109.14	82.23	77.41	52.09	40.76	36.87
Group 10	100	98.85	84.65	83.93	48.84	49.31	34.71
Group 11	100	98.40	79.00	74.31	52.52	51.19	40.92
Group 12	100	103.84	83.33	77.73	57.26	50.15	37.71
Mean	100	101.55	80.99	78.35	49.07	46.07	37.60
S.E.M	0	1.24	1.87	1.78	1.33	1.29	0.84

Table 3.2 : LDH release assay

	Control	6 hr	12 hr	18 hr	24 hr	36 hr	48 hr
Group 1	100	92.64	113.85	132.60	145.72	174.67	236.35
Group 2	100	97.76	116.26	138.95	165.58	176.28	228.82
Group 3	100	92.81	113.21	128.89	167.62	176.99	237.75
Group 4	100	99.53	110.68	124.37	147.47	200.23	247.29
Group 5	100	105.88	113.64	137.78	141.63	185.69	244.69
Group 6	100	108.13	118.49	122.46	145.59	183.26	246.86
Group 7	100	97.07	114.39	133.79	149.73	186.08	238.77
Group 8	100	102.65	114.71	138.40	164.31	184.47	238.77
Group 9	100	101.00	113.40	130.79	153.13	195.53	243.11
Group 10	100	93.86	114.05	135.82	149.24	187.72	241.66
Group 11	100	103.24	119.96	122.24	163.59	193.53	254.07
Group 12	100	104.05	112.95	128.51	150.63	188.35	243.80
Mean	100	99.89	114.63	131.22	153.69	186.07	241.83
S.E.M	0	1.49	0.73	1.74	2.62	2.26	1.85

4. Effects of geldanamycin on low potassium-induced injuries in cultured cerebellar granule neurons. Determined by MTT reduction assay.

Table 4.1 : Cotreatment

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	52.72	59.51	52.45	51.56	65.08	59.65	59.58
Group 2	100	61.96	60.68	49.87	50.54	53.13	57.20	67.26
Group 3	100	66.38	62.66	61.87	50.21	53.95	62.85	63.35
Group 4	100	61.16	82.54	62.36	52.12	54.17	54.80	62.66
Group 5	100	67.41	46.31	51.40	52.45	60.00	54.97	59.96
Group 6	100	60.28	49.74	52.24	50.84	57.62	56.92	60.03
Group 7	100	55.46	64.43	51.16	55.71	67.37	53.99	64.20
Group 8	100	66.92	55.21	62.11	59.72	54.89	60.03	65.95
Mean	100	61.54	60.14	55.43	52.89	58.28	57.55	62.87
S.E.M	0	1.91	3.89	1.98	1.15	1.92	1.09	1.02

Table 4.2 : Pretreatment with GA for 6 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	60.80	69.02	65.68	70.37	78.34	86.51	84.38
Group 2	100	71.98	67.29	70.89	72.24	77.63	86.18	86.05
Group 3	100	64.34	66.32	73.39	73.99	80.83	86.86	88.47
Group 4	100	72.39	64.51	70.24	71.85	80.43	90.42	89.41
Group 5	100	64.47	59.46	75.53	73.22	83.03	86.84	89.47
Group 6	100	76.97	53.63	71.84	71.05	80.79	87.30	88.62
Group 7	100	69.91	56.37	78.21	77.19	83.07	94.13	95.75
Group 8	100	54.82	61.33	73.14	73.28	86.87	91.50	91.64
Mean	100	66.96	62.24	72.37	72.89	81.37	88.72	89.22
S.E.M	0	2.54	1.94	1.32	0.75	1.04	1.04	1.22

Table 4.3 : Pretreatment with GA for 12 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	73.57	68.52	81.16	81.89	92.50	95.76	98.61
Group 2	100	77.65	57.40	77.57	80.02	90.70	94.54	102.94
Group 3	100	53.25	66.23	67.89	72.56	87.57	95.19	94.01
Group 4	100	68.49	71.18	67.61	66.72	81.66	86.39	87.43
Group 5	100	63.80	50.72	73.70	78.08	92.53	98.54	92.29
Group 6	100	70.45	53.54	73.21	75.24	93.1	101.85	100.32
Group 7	100	75.85	56.66	76.73	79.71	91.22	97.81	111.27
Group 8	100	78.90	50.26	77.54	78.90	92.59	103.86	106.92
Mean	100	70.25	59.31	74.43	76.64	90.23	96.74	99.22
S.E.M	0	3.01	2.91	1.69	1.76	1.38	1.87	2.78

Table 4.4 : Pretreatment with GA for 24 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	92.29	66.94	62.19	87.90	115.93	129.84	129.84
Group 2	100	60.88	81.24	66.03	90.93	109.27	121.57	126.92
Group 3	100	62.03	68.89	66.98	101.43	127.59	134.55	133.01
Group 4	100	57.39	69.44	67.75	100.33	122.29	135.10	141.06
Group 5	100	64.17	57.14	66.34	84.06	114.96	114.76	127.56
Group 6	100	51.57	43.69	63.19	87.99	108.46	111.61	125.98
Group 7	100	51.07	62.47	52.99	81.45	120.52	119.76	126.18
Group 8	100	52.14	44.69	63.03	92.95	123.08	123.82	125.54
Mean	100	57.69	61.81	63.56	90.88	117.76	123.87	129.51
S.E.M	0	1.91	4.54	1.67	2.53	2.40	3.08	1.87

Table 4.5 : Pretreatment with GA for 48 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	40.42	78.58	92.20	100.00	108.89	114.25	117.97
Group 2	100	40.99	76.69	90.38	96.28	108.26	113.16	119.06
Group 3	100	41.95	83.57	88.03	96.93	112.06	117.72	122.41
Group 4	100	45.54	88.27	88.19	98.95	112.46	117.39	121.68
Group 5	100	51.11	51.95	96.49	104.61	119.74	124.63	123.80
Group 6	100	43.46	53.13	96.86	102.12	118.91	127.68	124.63
Group 7	100	40.93	62.76	92.90	109.26	112.76	133.34	129.12
Group 8	100	42.14	70.70	93.83	105.35	115.95	128.81	129.01
Mean	100	43.32	70.71	92.36	101.69	113.63	122.12	123.46
S.E.M	0	1.26	4.81	1.19	1.59	1.50	2.65	1.45

Table 4.6 : Pretreatment with GA for 72 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	68.88	65.07	83.48	93.85	105.63	109.26	112.59
Group 2	100	59.35	78.81	83.85	92.74	107.04	121.67	108.30
Group 3	100	44.77	75.26	93.03	110.91	122.67	123.45	131.66
Group 4	100	57.56	58.80	94.35	104.55	120.74	108.81	136.15
Group 5	100	65.18	68.22	85.74	97.59	102.40	106.11	108.95
Group 6	100	58.29	66.80	82.24	97.38	101.75	115.93	108.29
Group 7	100	60.12	64.37	91.38	102.67	114.63	116.64	127.31
Group 8	100	57.28	50.77	87.27	100.65	116.86	112.15	131.56
Mean	100	58.55	66.01	87.67	100.04	111.47	114.25	120.60
S.E.M	0	2.29	3.11	1.65	2.11	2.93	2.21	4.29

5. Effects of geldanamycin on low potassium-induced injuries in cultured cerebellar granule neurons. Determined by LDH release assay.

Table 5.1 : Cotreatment

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	176.59	133.24	135.83	124.51	123.13	141.50	132.57
Group 2	100	193.37	130.87	131.78	129.17	120.44	133.49	142.36
Group 3	100	178.61	168.94	134.02	134.29	122.62	149.41	133.12
Group 4	100	176.58	176.21	132.63	124.81	130.77	149.27	136.75
Group 5	100	192.88	148.84	136.78	137.99	135.55	150.93	134.16
Group 6	100	161.65	124.44	133.91	146.42	127.84	148.89	147.82
Group 7	100	155.76	121.42	135.35	135.62	121.78	135.99	137.47
Group 8	100	196.49	144.86	133.32	126.83	126.69	139.00	134.93
Mean	100	178.99	143.60	134.20	132.46	126.10	143.56	137.39
S.E.M	0	5.27	7.15	0.59	2.68	1.82	2.44	1.85

Table 5.2 : Pretreatment with GA for 6 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	136.67	108.04	105.19	106.22	89.65	87.61	85.61
Group 2	100	141.07	105.63	107.48	110.58	100.97	88.87	85.68
Group 3	100	142.16	106.24	117.03	108.45	108.18	98.47	84.45
Group 4	100	137.53	111.13	117.69	103.65	103.65	96.28	89.15
Group 5	100	125.81	131.38	100.79	101.97	98.20	92.89	75.71
Group 6	100	137.53	130.38	101.03	104.56	95.44	89.95	87.47
Group 7	100	125.94	124.50	113.45	112.87	108.08	101.67	88.97
Group 8	100	148.23	118.63	108.19	104.20	92.85	92.40	87.24
Mean	100	136.87	119.49	108.86	106.56	99.63	93.52	85.54
S.E.M	0	2.73	3.59	2.34	1.33	2.42	1.74	1.52

Table 5.3 : Pretreatment with GA for 12 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	155.24	158.04	151.31	149.86	129.19	116.99	105.31
Group 2	100	173.38	185.14	149.12	151.49	130.28	115.32	105.71
Group 3	100	162.99	183.80	150.75	156.28	132.98	116.79	101.41
Group 4	100	192.19	141.49	151.88	153.44	136.25	118.66	111.31
Group 5	100	171.43	160.07	144.19	142.95	127.54	106.92	109.53
Group 6	100	156.48	124.93	139.82	156.48	126.52	107.99	106.78
Group 7	100	156.27	123.15	155.68	158.97	113.32	109.56	106.35
Group 8	100	175.71	145.04	151.17	146.25	123.15	119.42	100.77
Mean	100	167.96	152.71	149.24	151.97	127.40	113.96	105.89
S.E.M	0	4.52	8.38	1.76	1.93	2.46	1.77	1.27

Table 5.4 : Pretreatment with GA for 24 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	129.34	172.57	120.64	84.23	80.26	74.82	72.54
Group 2	100	137.41	112.06	125.63	84.38	84.45	90.04	74.38
Group 3	100	194.56	133.11	122.09	91.49	79.36	84.23	69.66
Group 4	100	143.91	174.6	127.55	98.53	86.19	73.10	67.33
Group 5	100	131.21	177.68	128.39	83.33	84.87	78.10	70.35
Group 6	100	139.30	112.95	124.83	83.14	87.19	91.90	68.06
Group 7	100	175.20	134.84	123.13	90.98	82.35	79.83	65.48
Group 8	100	183.72	170.82	135.54	90.94	75.31	82.42	72.37
Mean	100	154.33	148.58	125.98	88.38	82.49	81.81	70.02
S.E.M	0	9.16	10.02	1.65	1.95	1.41	2.38	1.06

Table 5.5 : Pretreatment with GA for 48 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	159.60	153.87	152.47	128.53	109.83	112.32	95.21
Group 2	100	162.95	145.69	153.92	126.08	104.69	93.77	99.65
Group 3	100	147.08	140.69	142.33	126.58	107.75	94.35	101.69
Group 4	100	149.33	156.91	143.75	127.18	112.53	106.90	91.68
Group 5	100	146.24	144.29	142.02	120.86	94.24	96.19	94.87
Group 6	100	155.52	163.35	146.71	124.46	94.58	105.08	98.17
Group 7	100	157.11	156.47	158.83	118.95	108.94	100.63	103.73
Group 8	100	151.10	151.69	144.36	127.29	112.18	93.20	96.52
Mean	100	153.62	151.62	148.05	124.99	105.59	100.31	97.69
S.E.M	0	2.16	2.68	2.21	1.19	2.59	2.52	1.39

Table 5.6 : Pretreatment with GA for 72 hr of incubation

	Control	Low K^+	Trolox	0.01 μM	0.025 μM	0.05 μM	0.075 μM	0.1 μM
Group 1	100	163.79	191.64	231.24	209.54	198.55	188.11	183.59
Group 2	100	164.66	154.20	225.44	183.56	179.63	188.50	169.61
Group 3	100	171.79	182.93	221.75	204.65	182.06	194.86	168.54
Group 4	100	219.09	170.43	245.02	178.07	181.95	183.27	170.74
Group 5	100	218.20	208.68	205.11	170.89	188.61	191.73	171.78
Group 6	100	172.29	210.44	223.78	174.11	161.31	190.36	169.59
Group 7	100	210.24	210.93	226.66	192.43	197.97	199.12	167.37
Group 8	100	218.87	155.95	227.91	182.14	177.87	182.29	156.08
Mean	100	192.37	185.65	225.86	186.92	183.49	189.78	169.66
S.E.M	0	9.27	8.38	3.90	4.98	4.24	1.98	2.64

6. Effects of trolox on low potassium-induced injuries in cultured cerebellar granule neurons. Determined by MTT reduction assay.

Table 6.1 : Cotreatment

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	54.92	41.67	38.89	38.71	36.61	31.23
Group 2	100	58.10	41.76	37.90	39.16	35.04	33.87
Group 3	100	53.87	44.38	38.41	38.24	37.72	34.22
Group 4	100	61.61	42.47	39.53	40.31	37.80	35.08
Group 5	100	57.74	45.78	44.62	43.57	41.67	37.24
Group 6	100	56.51	44.73	43.78	43.13	41.88	37.13
Group 7	100	48.29	50.16	43.76	46.27	47.49	47.00
Group 8	100	51.21	50.24	44.41	47.25	46.84	39.12
Mean	100	55.28	45.15	41.41	42.08	40.63	36.86
S.E.M	0	1.48	1.22	1.05	1.23	1.65	1.68

Table 6.2 : Pretreatment with trolox for 24 hr of incubation

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	60.19	63.10	62.45	66.29	58.89	54.07
Group 2	100	61.29	62.29	68.08	67.83	69.50	63.84
Group 3	100	59.32	68.67	70.54	64.54	76.76	66.79
Group 4	100	61.01	58.11	58.81	56.80	57.29	54.62
Group 5	100	59.69	58.61	60.93	58.81	69.06	62.99
Group 6	100	56.20	63.52	66.05	62.65	74.86	67.63
Group 7	100	51.64	59.05	66.04	69.28	67.03	68.94
Group 8	100	48.60	62.74	60.93	71.50	66.88	68.96
Mean	100	57.24	62.01	64.23	64.71	67.53	63.48
S.E.M	0	1.67	1.22	1.44	1.79	2.41	2.14

Table 6.3 : Pretreatment with trolox for 48 hr of incubation

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	44.62	37.82	40.77	40.38	45.51	47.69
Group 2	100	47.69	43.72	41.41	43.61	46.67	48.46
Group 3	100	47.75	45.50	45.29	41.65	41.86	42.39
Group 4	100	41.11	43.79	42.93	44.86	41.44	42.83
Group 5	100	39.67	44.48	46.63	43.25	44.68	41.51
Group 6	100	40.49	45.19	45.81	46.83	44.38	45.19
Group 7	100	64.09	44.84	46.08	45.29	46.79	48.20
Group 8	100	60.49	44.65	44.11	46.06	44.53	42.74
Mean	100	48.24	43.75	44.13	43.99	44.48	44.88
S.E.M	0	3.27	0.87	0.78	0.78	0.69	1.02

Table 6.4 : Pretreatment with trolox for 72 hr of incubation

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	60.96	53.59	54.60	58.11	57.37	57.55
Group 2	100	68.51	53.96	58.01	61.23	54.97	63.54
Group 3	100	51.94	51.52	52.28	54.64	52.87	55.57
Group 4	100	44.69	55.31	54.05	52.36	55.56	54.89
Group 5	100	68.74	56.31	61.84	62.62	64.37	67.09
Group 6	100	58.06	59.22	58.74	59.61	62.43	60.29
Group 7	100	61.78	60.15	51.96	54.78	60.67	59.81
Group 8	100	58.53	58.96	50.26	56.66	59.66	57.89
Mean	100	59.15	56.13	55.22	57.50	58.49	59.58
S.E.M	0	2.84	1.09	1.39	1.25	1.40	1.45

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7. Effects of trolox on low potassium-induced injuries in cultured cerebellar granule neurons. Determined by LDH release assay.

Table 7.1 : Cotreatment

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	148.49	171.81	174.19	165.45	188.38	165.69
Group 2	100	163.02	155.39	177.89	175.80	182.61	176.29
Group 3	100	167.53	171.13	155.58	158.89	171.33	163.61
Group 4	100	159.56	152.48	172.49	161.47	164.82	170.02
Group 5	100	155.76	177.63	169.51	155.69	165.84	158.97
Group 6	100	158.01	181.74	163.23	162.07	159.65	177.58
Group 7	100	165.28	166.13	161.78	164.29	171.35	181.78
Group 8	100	163.55	181.06	185.07	175.21	168.83	144.62
Mean	100	160.15	169.67	169.97	164.86	171.60	167.32
S.E.M	0	2.16	3.91	3.37	2.56	3.36	4.23

Table 7.2 : Pretreatment with trolox for 24 hr of incubation

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	139.20	126.36	121.18	123.60	122.29	123.16
Group 2	100	131.87	125.19	120.17	126.93	130.25	125.94
Group 3	100	137.38	105.05	119.96	117.01	124.11	134.45
Group 4	100	123.29	125.89	131.53	129.83	123.49	119.09
Group 5	100	121.72	127.23	124.76	122.64	142.48	130.36
Group 6	100	125.36	134.28	127.13	118.94	130.29	126.92
Group 7	100	127.10	129.47	130.03	130.55	123.33	122.49
Group 8	100	138.29	126.56	139.54	126.03	121.49	129.99
Mean	100	130.53	125.01	126.79	124.44	127.22	126.55
S.E.M	0	2.51	3.03	2.39	1.71	2.48	1.76

Table 7.3 : Pretreatment with trolox for 48 hr of incubation

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	273.26	204.60	202.34	198.38	198.96	200.75
Group 2	100	270.04	198.56	203.15	195.45	199.75	203.29
Group 3	100	221.02	205.10	199.83	185.47	202.44	193.38
Group 4	100	203.42	195.69	205.23	198.31	198.21	198.66
Group 5	100	196.12	191.36	199.26	190.14	194.05	200.94
Group 6	100	205.08	178.71	189.80	197.00	188.49	201.18
Group 7	100	223.63	191.20	191.73	189.83	205.93	183.95
Group 8	100	226.13	195.27	194.46	192.62	190.64	190.00
Mean	100	227.34	195.06	198.23	193.40	197.31	196.52
S.E.M	0	10.37	2.99	1.99	1.65	2.08	2.39

Table 7.4 : Pretreatment with trolox for 72 hr of incubation

	Control	Low K^+	10 μM	25 μM	50 μM	75 μM	100 μM
Group 1	100	279.38	269.17	245.23	224.83	256.76	246.01
Group 2	100	256.65	273.94	330.71	317.29	271.72	262.31
Group 3	100	202.33	282.48	282.37	257.64	262.97	254.44
Group 4	100	319.14	255.98	288.24	252.99	275.83	270.51
Group 5	100	287.94	261.99	266.44	295.28	301.83	286.50
Group 6	100	266.13	271.55	339.97	287.54	298.68	314.55
Group 7	100	260.60	301.17	283.87	318.88	300.26	269.33
Group 8	100	242.46	289.38	297.37	319.92	310.87	295.68
Mean	100	264.33	275.71	291.78	284.29	284.87	274.92
S.E.M	0	12.09	5.21	11.05	12.62	7.22	8.02

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Table 8. Effects of pretreatment with geldanamycin and trolox on glutathione level in low potassium-medium on cultured cerebellar granule neurons. Determined by glutathione assay.

	Control	Low K^+	GA 0.1 μM	GA+ low K^+	Trolox 100 μM	Trolox+ low K^+
Group 1	100	66.67	133.33	144.54	150.00	50.83
Group 2	100	58.34	125	137.14	133.33	59.71
Group 3	100	49.23	123.75	131.27	150.00	66.67
Group 4	100	68.12	124.77	139.73	166.67	71.22
Group 5	100	66.22	128.19	133.31	166.67	72.36
Group 6	100	63.16	119.23	130.66	166.67	81.38
Group 7	100	50.00	109.23	124.99	150.00	84.39
Group 8	100	66.67	125.00	140.74	150.00	78.32
Mean	100	61.05	123.56	135.29	154.17	70.61
S.E.M	0	2.72	2.49	2.26	4.17	3.99

Table 9. Effects of pretreatment with geldanamycin and trolox on low potassium-medium in cultured cerebellar granule neurons. Determined by TBARS assay.

	Control	Low K^+	GA 0.1 μM	GA+ low K^+	Trolox 100 μM	Trolox+ low K^+
Group 1	100	115.04	79.94	98.10	48.68	82.29
Group 2	100	138.57	69.01	107.27	48.17	82.82
Group 3	100	133.31	56.57	101.04	55.76	89.53
Group 4	100	117.19	79.28	88.35	51.87	81.56
Group 5	100	149.28	78.59	96.37	60.10	90.49
Group 6	100	135.98	78.53	83.01	74.64	89.62
Group 7	100	133.82	83.88	88.49	61.17	80.09
Group 8	100	122.76	88.94	77.53	70.06	79.43
Mean	100	130.74	76.84	92.52	58.81	84.48
S.E.M	0	4.10	3.51	3.49	3.43	1.63

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