Planta Medica

Natural Products and Medicinal Plant Research

Editor
Prof. Dr. Adolf Nahrstedt
Institut für Pharmazeutische Biologie
und Phytochemie der Universität
Hittorfstraße 56
D-48149 Münster
Germany
Fax: +49 251 83-383 41
E-mail: nahrste@uni-muenster.de

Georg Thieme Verlag
Stuttgart · New York
Rüdigerstraße 14
D-70469 Stuttgart
Postfach 30 11 20
D-70451 Stuttgart

Thieme New York
333 Seventh Avenue
New York, NY 10001

Pulsatilloside A and Anemoside A_3 Protect PC12 Cells from Apoptosis Induced by Sodium Cyanide and Glucose Deprivation

Xiang-Dong Gao[1, 2], Wen-Cai Ye[3], Albert C. H. Yu[4], Yun Zhang[3], Ren-Xiang Tan[2], Ming Li[1], W. L. Wendy Hsiao[4]

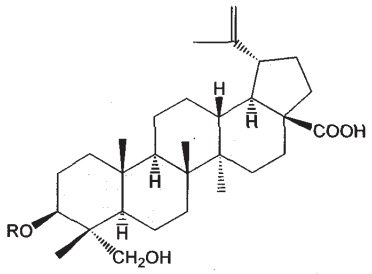

Fig. 1 Structures of pulsatilloside A (1) and anemoside A_3 (2).

1. R = α-L-arabinopyranosyl
2. R = α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl

Abstract

Using sodium cyanide (NaCN) and glucose deprivation induced cell injury in PC12 as an injury model, we investigated the protective effects of pulsatilloside A and anemoside A_3 on neurons. The results showed that PC12 cells under the NaCN-injury and glucose deprivation would undergo apoptosis. Additions of pulsatilloside A and anemoside A_3, at dosages ranging from 0.1, 1 and 10 μg/ml, protected PC12 cells from apoptosis determined by MTT, LDH release analysis, and flow cytometry measurement.

Abbreviations

β-AP: β-amyloid protein
FASC: flow cytometry
LDH: lactate dehydrogenase
NGF: nerve growth factor
TEM: transmission electron microscope

Recent findings demonstrated that apoptosis is involved in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD) and neuronal trauma [1]. In the case of AD, massive loss of neurons in the cerebral cortex and hippocampus is associated with deterioration of cognition and memory. Thus, the inhibition or prevention of apoptosis in these neurons would have therapeutic values to AD. Agents preventing apoptosis would have potential for the treatment of AD. The roots of *Pulsatilla chinensis* (Bunge) Regal have been used as a remedy in the traditional Chinese medicine for the treatment of cancer, amoebic dysentery, and bacterial infections [2]. We have previously isolated several lupane-type triterpene glycosides from this plant and elucidated their chemical structures [3], [4]. During the process of searching for anti-apoptotic reagents, we found that the total saponin fration of this plant showed some neuroprotecting activity. The bioassay guided fractionation of the total saponin resulted in the isolation of two active components, pulsatilloside A and anemoside A_3. In this paper, we reported the protective effects of these two lupane-type triterpenoid saponins on PC12 cells injured by NaCN and glucose deprivation.

The effect of the drugs on cell viability was determined by MTT assay. Compared with control normal cells, the survival rate of PC12 dropped down to 74% upon treatment with NaCN and glucose-deprivation (in brief, NaCN/glu injury) ($P < 0.01$) (Table **1**). However, exposure to pulsatilloside A significantly reduced the NaCN/glu-toxic effect ($P < 0.05$). At 10 μg/ml, pulsatilloside A completely protected PC12 from NaCN/glu injury. Similar result was also obtained with PC12 cell treated anemoside A_3. Together, these results indicated that pulsatilloside A and anemoside A_3 could increase cell viability significantly. In the same experiment, we included both NGF and ginsenoside Rg1 as positive control. Both molecules are known to act as neuroprotectant [5], [6]. The degree of cell injury was also examined by the activity of LDH released into the growth medium using the LDH activity assay kit. The LDH in the medium was increased by 88% after cell injury, compared with normal control (Table **2**). Addition of either pulsatilloside A and anemoside A_3 to the growth medium reduced the LDH release in a dosage dependent manner as shown in Table **2**.

TEM analysis showed that the injured PC12 cells exhibited characteristic apoptotic features – cell shrinkage, nuclear chromatin condensation and aggregation inside of the nuclear envelope. In the initial stage of apoptosis, the cell membrane remained intact and organelles were basically normal. In the later stage, the nuclear envelope invaginated and formed digitate process wrapping nuclear materials, which diffused to the cytoplasm and eventually formed apoptotic body (Fig. **2B**). In normal cells, both cell membrane and nuclear envelope are intact, and their nucleoplasm is well distributed (Fig. **2A**). Addition of the drugs increased the ratio of normal to apoptotic cells in the cultures. The quantification of the NaCN/glu induced apoptosis, as well as the protective effect of the drugs to the injured PC12 cells were performed using flow cytometry method. Fig. **3** illustrated the changes in DNA content detected by flow cytometry. A sharp sub-G_1 apoptosis peak appeared after exposure PC12 cells to NaCN/glu (Fig. **3B**). The percentage of apoptotic cells in this group is statisticlaly higher than the normal control (Table **3**). The result showed that the treatment of PC12 cells with NaCN and glucose-deprivation could induce apoptosis. Pulsatilloside A and anemoside A_3 treatments at 10 μg/ml effectively reduced the apoptotic peak of the distribution of DNA content (Fig. **3C** & **D**). The percentage of apoptotic cells was clearly reduced with these treatments (Table **3**).

Affiliation: [1] School of Biopharmaceutical, China Pharmaceutical University, Nanjing, China · [2] Institute of Functional Biomolecule, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Science, Nanjing University, Nanjing, China · [3] Department of Phytochemistry, China Pharmaceutical University, Nanjing, China · [4] Department of Biology, The Hong Kong University of Science and Technology, Hong Kong, China

Correspondence: W. L. Wendy Hsiao · Department of Biology · The Hong Kong University of Science and Technology · Kowloon, Hong Kong · E-mail: bowhsiao@ust.hk · Fax: 852-23581559

Received: May 21, 2002 · **Accepted:** September 22, 2002

Bibliography: Planta Med 2003; 69: 171–174 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943

Table 1 Protective effect of pulsatilloside A and anemoside A_3 on PC12 cells injury induced by NaCN/glucose deprivation, assessed by MTT assay

Group		$O.D._{570\,nm} \pm S.E.M.^{\phi}$	Survival (%)
Control (normal) cells		1.15 ± 0.18	100.0%
Control cells + pulsatilloside A,	10.0 μg/ml	1.14 ± 0.22	99.2%
Injured cells		0.85 ± 0.06#	73.9%
Injured cells + NGF,	5.0 ng/ml	1.08 ± 0.11**	93.9%
Injured cells + Rg_1,	10.0 μg/ml	1.08 ± 0.21	95.7%
Injured cells + pulsatilloside A,	0.1 μg/ml	0.99 ± 0.08*	86.1%
	1.0 μg/ml	1.07 ± 0.27**	93.0%
	10.0 μg/ml	1.17 ± 0.27**	101.7%
Control (normal) cells		1.39 ± 0.16	100.0%
Control cells + anemoside A_3,	10.0 μg/ml	1.40 ± 0.29	100.7%
Injured cells		0.98 ± 0.08#	70.5%
Injured cells + anemoside A_3	0.1 μg/ml	1.18 ± 0.17*	84.9%
	1.0 μg/ml	1.27 ± 0.19**	91.4%
	10.0 μg/ml	1.34 ± 0.07**	96.4%

Each experiment was conducted in 6 replicate wells. NGF (nerve growth factor) and Rg_1 ginsenoside are included as positive control. The data represented the average of 5 independent experiments. ϕ = $O.D._{Experimental} - O.D._{blank}$. The cell-free blank contained the MTT reaction mix with the designated test compound. #: $P < 0.01$, vs Control; *: $P < 0.05$, vs Injured cells; **: $P < 0.01$, vs Injured cells.

Table 2 Inhibitory effect of pulsatilloside A and anemoside A_3 on LDH release of PC12 cells under NaCN/glucose deprivation treatment

Group		LDH activity unit/ml ± S.E.M.	Increment (%)
Control (normal) cells		71.4 ± 5.03	–
Control cells + pulsatilloside A,	10.0 μg/ml	70.9 ± 4.11	–
Control cells + anemoside A_3,	10.0 μg/ml	71.1 ± 6.01	–
Injured cells		134.4 ± 1.10#	88.2%
Injured cells + NGF,	50.0 ng/ml	95.4 ± 7.93*	33.6%
Injured cells + Rg_1		91.3 ± 5.39**	27.9%
Injured cells + pulsatilloside A,	0.1 μg/ml	114.1 ± 3.86	59.8%
	1.0 μg/ml	97.5 ± 8.75*	36.6%
	10.0 μg/ml	80.3 ± 5.13**	12.5%
Injured cells + anemoside A_3,	0.1 μg/ml	117.6 ± 5.70	64.7%
	1.0 μg/ml	100.6 ± 6.28*	40.9%
	10.0 μg/ml	85.6 ± 4.51**	19.9%

Each experiment was conducted in 6 replicate wells. Data represented the average of five independent experiments. #: $P < 0.01$, vs control; *: $P < 0.05$, vs Injured cells; **: $P < 0.01$, vs Injured cells.

Cyanide is known to be a blocker in electron transport chain of various biological functions. Under such insults, a series of metabolic stress responses can be triggered within the injured cells, which include the elevation of extracellular glutamate, LDH activity. The accumulation of glutamate causes toxic effects to cells. Meanwhile, glutamate activated NMDA receptor and opens its ion channel in cell membrane. The large influx of calcium into cells could cause cell death. this NaCN/glucose deprivation injury mimic the oxidative stress-related neurodegenerative disorder such as Alzheimer's disease, Down syndrome [7], [8]. Using PC12 as an *in vitro* model, Mills et al. [9] demonsrated that cyanide induced apoptotic oxidative stress. Treatment with antioxidants significantly reduced the incidence of cyanide-induced apoptosis. In this study, we used similar injury model to study the protective effects of pulsatilloside A and anemoside A_3 in PC12 cells. The results showed NaCN/glu insult caused cytotoxicity and elevation of LDH release in PC12 cells. Typical apoptotic features were observed by TEM and flow cytometry analysis. Additions of pulsatilloside A and anemoside A_3 effectively increased cell viability and decreased the level of LDH release, and lessened morphological changes due to apoptosis at concentrations ranging from 0.1 to 10 μg/ml, suggesting strongly that both drugs contain neuroprotective activity. Our study is in line with previous observations, in which the neuroprotective effects of dammarane derivatives, such as Rb_1, Rg_1 & Rg_3 were observed using rat cortical cells as a test system [10]. Our data provides an important groundwork for the further mechanistic study and the future neuroprotective drug development.

Materials and Methods

Pulsatilloside A {≥ 98% in purity; $[\alpha]_D^{20}$: +102.5° (c 0.15, CH_3OH)} and anemoside A_3 {≥ 99% in purity; $[\alpha]_D^{20}$: −6.0° (c 0.55, CH_3OH)} were purified from the roots of *P. chinensis* [3],

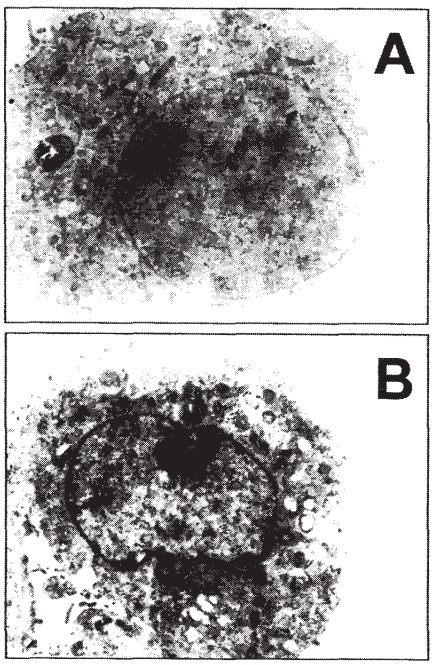

Fig. 2 Electron microscopic views of normal and apoptotic PC12 cells under NaCN/glucose deprivation treatment.

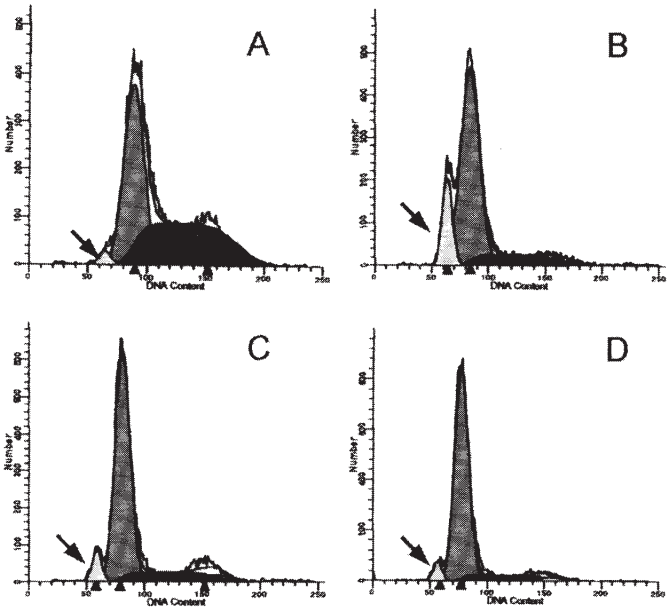

Fig. 3 Quantitative analysis of apoptotic cells of PC12 cultures in the presence and presence of drug treatments. Panel **A**: normal PC12 cells. Panel **B**: PC12 cells under NaCN/glucose deprivation (NaCN/glu) treatment. Panel **C** & **D**: NaCN/glu-injured PC12 treated with 10 μg/ml of pulsatilloside A and anemoside A_3 respectively. Arrow bar: sub-G_1 apoptotic peak.

[4], and were identified by comparing the IR, NMR, FABMS data and optical rotation values with those of authentic samples [3], [4]. PC12 cell line was grown in collagen-coated plates with DMEM medium supplemented with (v/v) 10% horse serum, 5% fetal bovine serum, and 1% glutamine. NaCN/Glucose-deprivation injury was conducted according to Li's mthod [11] with minor modifications. In brief, PC12 cells were grown in 24-well collagen-coated plates at 37 °C, in a humidified incubator with 5% CO_2. Confluent cultures were rinsed twice with Earle's medium free of glucose. Cells were then fed with glucose-free *medium with NaCN at the final concentration of 10 mM. Pulsa*-tilloside A and anemoside A_3 at designated dosages were then added to the test cultures and incubated for 25 min in aCO_2 incubator at 37 °C. After 25 min, the medium was removed. Cells were rinsed with D-Hanks solution twice and maintained in 0.5 ml DMEM medium for 24 hours. MTT assay was performed according to Mosmann's method [12]. The MTT reaction mixture on each well of the 96-well culture plate was measured using the ELISA reader DG-3022A at wavelength between 570 nm. The level of cell injury was determined by the activity of LDH released into medium using LDH activity assay kit (Nanjing Jiancheng Research Institute of Biotechnological Engineer, China). Apoptotic features fo the treated cells were examined under the transmission electron microscope (H-7000 TEM). PC12 cells were fixed, sliced and stained uranium-chromium dye according to Maunsbach [13] for TEM viewing. FASC analysis was applied to determine number of apoptotic cells with SACS Calabu 2A flow cytometer based on Hotz et al. [14]. A subG_1 peak proceeding the G_1 peak represents the apoptotic cell population.

Table 3 Quantitative analysis of apoptotic cell populations using flow cytometry method

Groups	*Apoptosis (%) + S.E.M.*
Control (normal cells)	2.01 ± 0.81
Injured cells	*18.70 ± 1.90**
Injured cells + Rg_1 (10 μg/ml)	7.05 ± 1.23**
Pulsatilloside A (10 μg/ml)	4.64 ± 0.96**
Anemoside A_3 (10 μg/ml)	6.36 ± 1.32**

Each experiment was done in triplicate. *: $P < 0.01$, vs Control; **: $P < 0.01$, vs Injured cells.

Acknowledgements

This work was supported by a grant from the Natural Science Foundation of Jiangsu Province (BK99096).

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Clionasterol: A Potent Inhibitor of Complement Component C1

Fátima Cerqueira[1], Rawiwan Watanadilok[2], Pichai Sonchaeng[2], Anake Kijjoa[1,3], Madalena Pinto[1], Henriette Quarles van Ufford[4], Burt Kroes[4], Cees Beukelman[4], Maria São José Nascimento[1]

Abstract

Clionasterol (**1a**), clionasterol monoacetate (**1b**) and 5α,8α-epidioxy-24α-ethylcholest-6-en-3-ol (**2**), isolated from the marine sponge *Xestospongia exigua*, and β-sitosterol (**3**) were tested for their influence on the classical (CP) and alternative (AP) pathways of activation of the human complement system *in vitro*. All the sterols inhibited the CP in a dose-dependent manner but no detectable effect was observed in the AP even at concentrations of 400 μM. Clionasterol was found to be a potent inhibitor of CP (IC_{50} = 4.1 μM) being ten-fold more active than β-sitosterol. The presence of the epidioxy group on C-5 and C-8 of compound **2** caused a pronounced decrease of the inhibitory effect. Mechanistic studies on the anticomplementary effect of clionasterol revealed that it interferes with the complement component C1.

Marine organisms have afforded a large number of 3β-hydroxysterols and their oxygenated analogues [1]. Of over 200 new sterols isolated, 5α,8α -epidioxysterols form a significant group [2]. We have isolated from the marine sponge *Xestospongia exigua*, collected from the Gulf of Thailand, clionasterol (**1a**) and 5α,8α-epidioxy-24α-ethylcholest-6-en-3-ol (**2**) and we have investigated their effect, together with that of β-sitosterol (**3**), on the CP and AP of human complement activation. Clionasterol and 5α,8α-epidioxy-24α-ethylcholest-6-en-3-ol have been previously isolated from the marine red alga, *Gracilaria edulis* [3] and from the marine sponge *Tethya aurantia* [2], respectively, but their biological activities have never been investigated. This is the first report of anticomplementary activity of marine sterols. The structures of **1a** and **2** were established by comparison of their MS, ^{1}H-, ^{13}C-NMR and rotation data with those of the authentic sample [4], as well as with those previously reported [3], [5].

The effects of **1a**, **1b**, **2** and **3** on the CP and AP of human complement activation are summarized in Table **1**. All the sterols studied

Affiliation: [1] Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto, Faculdade de Farmácia, Porto, Portugal · [2] Bangsaen Institute of Marine Science, Burapha University, Bangsaen, Chonburi, Thailand · [3] Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal · [4] Biogenic Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Utrecht, Utrecht, The Netherlands

Correspondence: Dr. M. S. J. Nascimento · Faculdade de Farmácia · Universidade do Porto · Rua Aníbal Cunha 164 · 4050–047 Porto · Portugal · Fax: +351-222003977 · E-mail: saojose@ff.up.pt

Received: July 12, 2002 · **Accepted:** November 16, 2002

Bibliography: Planta Med 2003; 69: 174–176 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943