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Original Article

Clivorine, an otonecine pyrrolizidine alkaloid from *Ligularia* species, impairs neuronal differentiation via NGF-induced signaling pathway in cultured PC12 cells

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ABSTRACT

Background: Pyrrolizidine alkaloids (PAs) are commonly found in many plants including those used in medical therapeutics. The hepatotoxicities of PAs have been demonstrated both in vivo and in *vitro;* however, the neurotoxicities of PAs are rarely mentioned.
Purpose: In this study, we aimed to investigate in vitro neurotoxicities of clivorine, one of the PAs found in various *Ligularia* species, in cultured PC12 cells.
Study design: PC12 cell line was employed to first elucidate the neurotoxicity and the underlying mechanism of clivorine, including cell viability and morphology change, neuronal differentiation marker and signaling pathway.
Methods: PC12 cells were challenged with series concentrations of clivorine and/or nerve growth factor (NGF). The cell lysates were collected for MTT assay, trypan blue staining, immunocytofluorescent staining, qRT-PCR and western blotting.
Results: Clivorine inhibited cell proliferation and neuronal differentiation evidenced by MTT assay and dose-dependently reducing neurite outgrowth, respectively. In addition, clivorine decreased the level of mRNAs encoding for neuronal differentiation markers, e.g. neurofilaments and TrkA (NGF receptor). Furthermore, clivorine reduced the NGF-induced the phosphorylations of TrkA, protein kinase B and cAMP response element-binding protein in cultured PC12 cells.
Conclusion: Taken together, our results suggest that clivorine might possess neurotoxicities in PC12 cells via down-regulating the NGF/TrkA/Akt signaling pathway. PAs not only damage the liver, but also possess neurotoxicities, which could possibly result in brain disorders, such as depression.

Introduction

Pyrrolizidine alkaloids (PAs) are common toxic agents found in about 3% of the world's flowering plants. Until now, more than 600 PAs have been found in nature, especially those from the families of Asteraceae, Boraginaceae and Fabaceae: these plants are commonly used as herbal medicine in the treatment of traumatic injury, pain, inflammation and other ailments (Roeder, 1995 and 2000). Unfortunately, PAs possess serious toxicity: because they contain an unsaturated 1, 2-double bond in the necine base, one of the most toxic compounds of plant origin. More importantly, PAs have been detected in many food products, such as honey and milk. The toxic substances, therefore, may be transferred to

Abbreviations: Akt, protein kinase B; CREB, cAMP response element-binding protein; DHP, dehydro-PA; Erk, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NGF, nerve growth factor; NF, neurofilaments; PA, pyrrolizidine alkaloid; PARP, poly ADP ribose polymerase; TrkA, tyrosine kinase receptor A.

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humans through the food chain causing intoxication (Goeger et al., 1982; Medeiros et al., 1999; Dübecke et al., 2011). As the result, special attention has been paid on the safety of PA worldwide (World Health Organization, 1989; German Federal Health Bureau, 1992; Pharmacopoeia of the People's Republic of China, 2010). Despite PAs are primarily considered as hepatotoxic, an extra-hepatoxic effect, such as pneumotoxic and neurotoxic, was also related to its intoxication in animals (Lee et al., 2005; Cooper et al., 1999).

Clivorine is an otonecine PA found in the plants from various *Ligularia* species (Asteraceae), including *Ligularia hodgsonii* and L. *wilsoniana*. Both species are commonly called Chuanziwan, which are commonly used as erroneously substitute of an herbal medicine Asteris Radix et Rhizoma (commonly called Ziwan, the dried root and rhizome of *Aster tataricus*) for antitussive function. The recommended dosage of Ziwan intake should be 5–10 g per day (Pharmacopoeia of the People's Republic of China, 2010). Considering about 0.8 mg/g clivorine found in Chuanziwan (Cheng et al., 2011), over 4 mg of clivorine should be taken by usage of the herb; this dose is 4000-fold higher than the safety limit of PA according to German Federal Health Bureau, i.e. 1 μg/day (German Federal Health Bureau, 1992). Therefore, it should be a great threaten to human health. Multiple administrations of Chuanziwan water extracts could cause significant liver injury with elevated serum ALT and AST activities in rats (Cheng et al., 2011). In clivorine-induced hepatotoxicity, the activations of growth-related kinase and caspase signaling pathways, as well as the oxidation system, were revealed (Ji et al., 2002, 2005, and 2010). However, very limited study has been done on the neurotoxicity of clivorine. Owing to its unique structure, clivorine exists in either a lipophilic non-ionized form or a hydrophilic ionized form (Lin et al., 2000): the unique dual solubilities may influence its toxicity by enhancing penetration via blood-brain barrier. On the other hand, clivorine showed toxicity on non-hepatic cells without P450 enzymes, such as human embryonic kidney 293 (HEK293) cells (Ji et al., 2002 and 2008). Thus, clivorine should be an excellent candidate for investigating the extra-hepatic toxicity on non-hepatic cell models, e.g. neuron.

PC12 cell is a rat pheochromocytoma cell line. Although they are not considered adult neurons, they undergo changes in phenotype exhibited by normal neurons when treated with nerve growth factor (NGF), including outgrowth and extension of neurites and maintenance of the structural basis of neural communication. Thus, PC12 cell model has been accepted as a widely used model for studying of neuronal properties and neurotoxicity in vitro (Greene et al., 1987). The challenge of clivorine in PC12 cultures showed robust decrease of markers for neuronal differentiation, including (i) NGF-induced neurite outgrowth; (ii) mRNAs and proteins encoding for neurofilaments; and (iii) downstream signaling of NGF, i.e. phosphorylation of TrkA, protein kinase B (Akt) and cAMP response element-binding protein (CREB).

Material and methods

Chemicals and reagents

Clivorine was purchased from Shanghai R&D Centre for Standardization of Traditional Chinese Medicine (>98% purity; Shanghai, China; molecular weight of 405 Da). The ^{13}CNMR spectrum of clivorine was run on a Bruker AVANCE-III instrument operating at 600 MHz with tetra-methylsilane (TMS) as internal standard and compared with those reported (Tan et al., 2001) (Suppl. Table 1). Clivorine was dissolved in dimethyl sulfoxide (DMSO) forming 100 mM stock solution and stored at −20 °C, and the final concentration of DMSO in all the culture medium was kept at 0.1%. The roots of L. *hodgsonii* were collected from Hehuachi Market (Sichuan, China) and authorized by the authors. The water extract of herb was prepared by boiling with water according to our reported method (Xiong et al., 2011 and 2014), and the content of clivorine was determined to be about 2.10 mg/g using an UPLC-MS system (Supplementary Text 1). Proper amount of extract was dissolved in water by ultra-sonication to make a stock solution of 250 mg/ml, which equaled to 1.27 mM of clivorine, and stored at −20 °C.

Cell cultures

Rat pheochromatocytoma PC12 cell was obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% fetal bovine serum, 6% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified at 37 °C with 7.5% CO_2. Fresh medium was supplied every other day. All culture reagents were purchased from Invitrogen Technologies (Carlsbad, CA). Experiments were performed on cells that had undergone fewer than fifteen passages.

Cell viability

After being treated with clivorine, the cell viability was measured by the colorimetric 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were cultured in 96-well plate and treated with series concentrations of clivorine and water extracts of L. *hodgsonii* root. After the drug treatment for 48 h, MTT solution (0.5 mg/ml) was added into the cultures and then incubated for additional 3 h. After the medium was removed, DMSO was added into each well before measuring the absorbance at 570 nm. The cell viability was expressed as the percentage of absorbance value of control (DMSO treatment), where the absorbance value was set as 100%. Besides, the cell viability was also measured by trypan blue staining according to the reported method (Ji et al., 2008). Cells were incubated with clivorine for 48 h before trypsinization; then the cells were mixed with 0.4% trypan blue-PBS for 2 min, and the dead cells were stained blue by trypan blue. The number of stained and unstained cells was counted using a hemocytometer.

Immunocytofluorescent staining

PC12 cells were grown on glass cover-slip. After being treated with NGF and series concentrations of clivorine for 48 h, the cells were then fixed with 4% paraformaldehyde (PFA) in PBS for 15 min, followed by 50 mM ammonium chloride (NH_4Cl) treatment for 25 min. Cultures were permeablized by 0.1% Triton X-100 in PBS for 10 min and blocked by 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Anti-NF68 primary antibody (1:500) was then applied on the cells for 16 h at 4 °C. Then, cells were stained with Alexa 555-conjugated secondary antibody (1:1000) and diamidino-phenyl-indole (DAPI; 5 μg/ml; Sigma-Aldrich, St Louis, MO) for 1 h at room temperature. After being washed with PBS for 4 times in 1 h, the cells were dehydrated serially with 50%, 75%, 95% and 100% ethanol and mounted with fluorescence mounting medium. Samples were then examined by ZEISS LSM710 Laser Scanning Confocal Microscope (Zeiss Inc., Jena, Germany). Images were captured with Ex 405 / Em 400–515 nm for DAPI (blue color) while Ex 560 / Em 560–695 nm for NF68 (red color).

Determination of neurite outgrowth

The determination of neurite outgrowth in PC12 cell cultures was performed according to a previous paper (Xu et al., 2012). PC12 cells (5×10^4 cells/well) were seeded onto 6-well plates