Cytotoxicity and L-Amino Acid Oxidase Activity of Crude Insect Drugs

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The cytotoxicity of crude insect drugs was measured using HeLa cells originating from human cervix and uterine cancer, using the dye uptake assay in order to find potential anticancer agents. Three kinds of extracts (buffer, methanol and ethylacetate) were prepared from 26 insects and used as raw materials for the activity assay. Among these, the buffer extracts from Tabanus, Mylabris and Huechys showed a potent anticancer activity, and those from Catharsius, Red ant, Scorpion, Tabanus and Vespae Nidus showed a strong L-amino acid oxidase (AAO) activity as well as cytotoxicity. In contrast, buffer extracts from *Gryllotalpa orientalis* and *Apriona germari* larvae showed greater/more rapid Hela cell growth than that of other insects.

Key words: Crude insect drugs, Cytotoxic activity, L-Amino acid oxidase

INTRODUCTION

Venoms from numerous invertebrates including insects and marine animals, as well as land snakes have been shown to possess cytotoxic or lytic effects on tumor cells *in vitro* (Newman *et al.*, 1993). In the case of insects, there have been many such reports. The lytic peptide cecropins, have been isolated from the haemolymph of the giant silk moth, *Hyalophora cecropia* and showed anticancer activities *in vitro* (Moore *et al.*, 1996). Norcantharidin is a demethylated form of cantharidin, and is an active ingredient of the blister beetle, Mylabris, and it is known to possess significant anti-hepatoma activity (Mack *et al.*, 1996), as it inhibited K562 human myeloid leukemia cells *in vitro* (Yi *et al.*, 1991). Such venom compounds may be developed as potential anticancer agents. Known components of insect toxins that are cytotoxic to cancer cells include, phospholipase A_2 (PLA_2) (Bomalaski *et al.*, 1995; Martikainen *et al.*, 1993), phospholipase C (PLC) (Markland, 1990), cytotoxins such as, pierisin from the pupae of the cabbage butterfly (Kono *et al.*, 1999), gallysin-1 from *Galleria mellonella* larvae (Bresfold *et al.*, 1997), pilosulin 1 from the venom of the jumper ant *Myrmecia pilosula* (Wu *et. al.*, 1998), and L-amino acid oxidase (AAO) (Stocker, 1990). PLA_2, PLC, and cytotoxins have direct lytic effects on cells, and their molecular weights are within the range 6,000 to 15,000 dalton. In contrast, an *in vitro* cytotoxic mechanism of AAO may be due to the generation of hydrogen peroxide causing cell death (Terada *et al.*, 1988). In our previous report, we elaborated upon the cytotoxic mechanism of AAO from king cobra venom, and upon the relationship between cytotoxicity and AAO activity of animal venoms (Ahn *et al.*, 1997A; Ahn *et al.*, 1997B). Here we report upon the cytotoxicity and AAO activity of the buffer extracts of twenty-six insects to further expand upon this relationship.

MATERIALS AND METHODS

Materials

The crude insect drugs; Catharsius, Ciacadae periostracum, Eupolyphaga, Hirudo, Huechys, *Mantidis oötheca*, Mylabris, Red ant, Scolopendra, Scorpion and Tabanus were purchased at a local market in Beijing, China, and others were supplied by the Department of Sericulture and Entomology, National Institute of Agricultural Science and Technology, Korea.

Preparation of Test Solution

Fifty grams of each insect crude drug were homogenized. The homogenates were extracted with 50 ml of

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40 mM Tris-HCl (pH 7.4) and the residue was soaked with methanol and ethyl acetate. Each supernatant was filtered with Whatman No. 6 filter paper and dried with a Hanil vacuum concentration system (Seoul, Korea). Each sample (10 mg) was dissolved in 500 µl of phosphate buffered saline (PBS), containing 0.5% ethanol or 0.5% DMSO.

Cell lines

The cell lines, SNU-1 (stomach cancer cell line: CRL 597.1) established by the Cancer Research Institute, College of Medicine, Seoul National University, Korea, and HeLa (cervix cancer cell line), were maintained under monolayer conditions in RPMI 1640 medium (GIBCO, New York, USA) supplemented with 10% newborn calf serum (GIBCO), 1 mmol/L L-glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin sulfate (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Measurement of Cytotoxicity

The cytotoxicities of the crude insect test solutions were tested against SNU-1 cell line using XTT{sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} kit solution (Boehringer Mannheim), as described previously (Geldof *et al.*, 1999). All measurements were performed in triplicate. The IC_{50} (50% inhibitory concentration; mg/ml) was defined as the fraction of extract that caused a 50% inhibition of cell viability compared with the control. We also determined the cell growth ratios of the insect fractions (Minoura *et al.*, 1995) to determine whether they inhibited or stimulated the growth of the two cell lines (HeLa and SNU-1), and the ratio of cell growth (Minoura *et al.*, 1995) of the two cell lines with respect to the different insect fractions using the XTT dye uptake method.

Table I. Measurement of cytotoxicity of crude insect drugs to HeLa cell line

Insect	Insect source	IC_{50} (mg/ml)		
		Buffer ex.	MeOH ex.	EtOAc ex.
Agrius convolvuli Larvae	*Agrius convolvuli* LINNAEUS	>100	5.6	9.9
Apriona germari Larvae	*Apriona germari* HOPE	>100	769.9	169.7
Bumble bee female (Worker)	*Bombus ignitus* SMITH	>100	0.5	>100
Bumble bee Larvae	*Bombus ignitus* SMITH	>100	16.3	>100
Bumble bee male (Drone)	*Bombus ignitus* SMITH	>100	8.1	>100
Gryllotalpa orientalis	*Gryllotalpa orientalis BURNMEISTER*	>100	0.3	4.1
Catharsius	*Catharsius molossus* L.	1.6	4.3	>100
Cicadae periostracum	*Cryptotympana atrula* FABR	0.2	4.7	4.1
Cordyceps	*Paecilomyces japonica*	6.8×10^{3}	0.6	>100
Dermestid beetles	*Trogoderma ternkton*	9.0	1.9	>100
Eupolyphaga	*Eupolyphaga sinensis* WALKER	49	41.5	>100
Harmonia axyridis	*Harmonia axyridis PALLAS*	10.4	0.3	>100
Hirudo	*Whitmania Pigra* WHITMAN	3.5	0.2	>100
Huechys	*Huechys sanguinea* DE GEER	4.0×10^{-4}	0.3	0.3
Larvae of Scarabaeoidea	*Protaetia brevitarsis seulensis KOLBE*	83	1.4	21.7
Lumbricus	*Pheretima aspergillum* E. PERR	>100	0.2	8.2
Mantidis oötheca	*Paratenodera sinensis* SAUSSURE	>100	1.2	2.5×10^{-2}
Mantidis oötheca	*Tenodera sinensis* SAUSSURE	>100	4.4	0.2
Mimela splendens	*Mimela splendens* GYLLENHAL	467	0.8	>100
Mylabris	*Mylabris phaelerata* PALL	7.3×10^{-2}	1.4×10^{-2}	0.1
Oxya japonica japonica	*Oxya japonica japonica* THUNBERG	31	1912	3606.8
Red Ant	*Formica rufa*	1.1×10^{-3}	0.4	0.2
Scolopendra	*Scolopendra morsitans* L.	80	0.3	>100
Scorpion	*Buthus martensi* KARSCH	8.0×10^{-4}	0.6	2.2
Scutelleride	*Poecilocoris lewisi* Distant	>100	0.5	1724
Silkworm adult male	Bombyx mori	>100	>100	17.5
Silkworm	Bombyx mori	>100	>100	1178.8
Tabanus	*Tabanus sp.*	1.8×10^{-2}	28.8	1.6×10^{-2}
Vespae Nidus	*Polistes mandarinus* SAUSS	0.1	1.1	1.0
Control	Mitomycin C (0.1 mg/ml)	7.6×10^{-2}		

L-Amino acid oxidase assay

L-Amino acid oxidase activity was determined spectrophotometrically with L-leucine as a substrate as described previously (Holme and Goldberg, 1975). One unit of activity was defined as the amount of oxidative deamination caused by 1.0 μ mole of L-leucine per min at pH 7.5 and 37°C.

Protein assay

Protein was measured by Coomassie blue R-250 using the Bradford method (Bradford, 1976). Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Cytotoxicity and L-amino acid oxidase activity of crude insect drugs

The cytotoxic activities of insect crude drugs were evaluated by XTT assay, results are shown in Table I. Of twenty-six crude insect drugs, twelve originated from oriental medicinal drugs and the remainder were from our institute. The IC_{50} values of five insect buffer fractions including Huechys, Mylabris, Red ant, Scorpion and Tabanus were lower than 1 mg/ml for HeLa cells. Cytotoxicity and L-amino acid oxidase activity results are shown in Table II. When cytotoxicity was compared with AAO activity, a strong correlation was found among samples of Catharsius, Huechys, Mylabris, Scorpion, Tabanus and Vespae Nidus. These showed both high cytotoxicity and AAO activity, but *Apriona germari* larvae, Bumblebee worker, Bumblebee drone and silkworm exhibited high AAO activity and weak cytotoxicity. These species contain other cytotoxic proteins; bee venom contains cytotoxic protein (mellitin) (Degrado, *et al.*, 1982) and silkworm an antibacterial protein (moricin) (Hara and Yamakawa, 1995), and should be further studied in terms of their cytotoxic components.

We examined the ratio of cell growth with respect to insect extracts to identify possible positive/negative cell growth factors. Insect fractions for example may not only inhibit cell proliferation but also promote cell growth. During the course of this work we found that the buffer extracts of *Gryllotalpa orientalis* and *Apriona germari* larvae promoted cell growth by a factor of two compared to the other insects examined (Table III).

Table II. Measurement of cytotoxicity and L-amino acid oxidase activity of the buffer extracts of insects to HeLa cell line

Insect	IC_{50} (mg/ml)	AAO activity(U/mg protein)
Agrius convolvuli Larvae	100>	trace
Apriona germari Larvae	100>	11.17
Bumble bee Worker	100>	12.02
Bumble bee Larvae	100>	trace
Bumble bee Drone	100>	13.38
Gryllotalpa orientalis	100>	trace
Catharsius	1.6	14.51
Cicadae periostracum	0.2	trace
Cordyceps	6.8×10^{-3}	trace
Dermestid beetles	9.0	trace
Eupolyphaga	49	trace
Harmonia axyridis	10.4	race
Hirudo	3.5	trace
Huechys	4.0×10^{-4}	8.36
Larvae of Scarabaeoidea	83	6.19
Lumbricus	100>	trace
Mantidis oötheca	100>	trace
Mimela splendens	467	trace
Mylabris	7.3×10^{-2}	8.00
Oxya japonica japonica	31	4.29
Red Ant	1.1×10^{-3}	4.46
Scolopendra	80	0.17
Scorpion	8.0×10^{-4}	11.78
Scutelleride	100>	1.10
Silkworm adult male	100>	trace
Silkworm	100>	11.25
Tabanus	1.8×10^{-2}	10.13
Vespae Nidus	0.1	13.95

Table III. Measurement of the ratio of cell growth to insect fractions (%)

Insect	HeLa			SNU-1		
	Buffer ex.	MeOH ex.	EtOAc ex.	Buffer ex.	MeOH ex.	EtOAc ex.
Agrius convolvuli Larvae	100	97	89	98	112	108
Apriona germari Larvae	191	112	111	73	106	91
Bumble bee Worker	140	105	94	81	94	104
Bumble bee Larvae	152	105	94	77	100	92
Bumble bee Drone	127	111	94	76	92	98
Catharsius	103	94	104	88	77	101
Cicadae periostracum	114	96	71	88	100	114
Cordyceps	136	90	117	86	83	57
Dermestid beetles	114	107	95	78	105	106
Eupolyphaga	116	113	94	101	90	119
Gryllotalpa orientalis	228	91	103	418	78	118

Table III. Continued.

Harmonia axyridis	98	91	87	73	69	87
Hirudo	116	55	104	79	61	85
Huechys	90	75	86	92	97	90
Larvae of Scarabaeoidea	93	98	88	91	90	88
Lumbricus	127	72	102	81	83	99
Paratenodera sinensis	121	84	63	107	90	106
Tenodera sinensis (p.mantis)	96	104	93	113	114	96
Mimela splendens	117	95	104	116	84	110
Mylabris	166	56	66	391	81	80
Oxya japonica japonica	138	105	77	77	97	93
Red Ant	71	50	98	87	81	85
Scolopendra	111	99	94	91	76	129
Scorpion	86	101	101	114	79	99
Scutelleride	138	79	93	76	98	130
Silkworm adult male	117	99	80	95	85	104
Silkworm	115	96	92	92	78	97
Tabanus	128	102	67	108	104	98
Vespae Nidus	135	102	90	108	78	126

*Growth ratio was defined as $100\times\frac{(S_t-S_0)}{(C_t-C_0)}$ where St and Ct are the absorbances for the test samples and the control, respectively, at incubation time t, and S_0 and C_0 are the absorbance of the blank test sample and the blank control, respectively.
*The concentration of each fraction was 0.1 mg/ml.

We have previously reported upon the L-amino acid oxidase activity of the venom of *Ophiophagus hannah* (king cobra) (Ahn *et al.*, 1997A, B). While microbial- and plant-derived products have been traditionally viewed as sources of novel anticancer agents, toxins of animal origin have received considerably less attention. The recent identification of insect toxins such as norcantharidin and pierisin (Kono *et al.*, 1999), which are known to be the inhibitors of protein phosphatase 2 A and apoptosis, respectively, suggested their potential use as anticancer agents. Some trials have been undertaken upon the inhibition of tumor cell growth with norcantharidin. It was found to have significant anti-hepatoma activity and was relatively free from side effects (Mack *et al.*, 1996). The venoms from 30 arthropods including 26 spider species were also assessed for cytotoxicity using Sf9 cells and three mammalian sources (murine neuroblastoma, macrophages and human osteosarcoma) (Cohen and Quistad, 1998). The most cytotoxic venoms to the four cell lines were from predatory jumping spiders (Salticidae, Phidippus sp.) and a centipede (Scolopenra sp.), which showed 50% growth inhibition responses of 1-8 μg venom per ml. The cytotoxicity of *Phidippus ardens* venom at these levels was instantaneous, as evidenced by a dramatic disruption of cell membranes and cell collapse. A lectin from the hemolymph of Sarcopha larvae, obtained after injury of their body wall, induced cytotoxic effects on tumor cells in the presence of murine macrophage (Nakajima and Natori, 1990; Itoh *et al.*, 1986A, B).

Our results demonstrate the extent of cytotoxicity and AAO activity variation in these insect buffer extracts. In case of some insects, a strong correlation was found between L-amino acid oxidase activity and cytotoxicity, but other venoms with low IC_{50} values (<1.0 mg/ml) should be further studied in terms of their cytotoxic components. Nevertheless, these results suggest that AAO may be one of the insect cytotoxic proteins and its properties should be further characterized in the near future.

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