L-amino acid oxidase จากพิษงูจงอางสามารถเหนี่ยวนำให้เกิดการตายแบบ apoptosis ในเซลล์มะเร็งผิวหนังมนุษย์

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บทคัดย่อ

L-amino acid oxidase (LAAO) เป็นส่วนประกอบหนึ่งในพิษงูที่มีความเป็นพิษต่อเซลล์ การศึกษาเกี่ยวกับสารต้านมะเร็งผิวหนังในประเทศไทยยังมีไม่มากนัก งูจงอางเป็นงูพิษที่มีขนาดใหญ่ ปริมาณน้ำพิษมีมาก การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของ LAAO จากพิษงูจงอางต่อเซลล์ผิวหนังปกติและเซลล์มะเร็งผิวหนัง โดยแยก LAAO จากพิษงูจงอางด้วยเทคนิคโครมาโทกราฟี ตามขนาดน้ำหนักโมเลกุล การใช้ตัวแลกเปลี่ยนประจุ และการจับกันอย่างจำเพาะ ทำการทดสอบ LAAO ในเซลล์ทั้งสองชนิด และตรวจวัดความเป็นพิษต่อเซลล์ด้วยวิธี MTT assay พบว่า ที่เวลา 48 ชั่วโมง LAAO จากพิษงูจงอางไม่ทำให้เซลล์ผิวหนังปกติตาย แต่ทำให้เซลล์มะเร็งผิวหนังตายประมาณร้อยละ 27 และจากการตรวจสอบ phosphatidyl serine ด้วยเครื่อง flow cytometry พบการตายของเซลล์ทั้งสองชนิดเป็นแบบ apoptosis โดยการตายของเซลล์เกี่ยวข้องกับ H_2O_2 ที่เกิดขึ้น ซึ่งเกิดจากปฏิกิริยาของ LAAO การใช้เอนไซม์ catalase ทดสอบสามารถป้องกันการตายของเซลล์ที่เกิดขึ้นจาก LAAO ได้ เมื่อตรวจสอบโปรตีนที่เกี่ยวข้องกับการตายแบบ apoptosis พบการย้าย cytochrome c จากไมโทคอนเดรียไปสู่ไซโตซอล และมีการแสดงออกของโปรตีน Bcl-2 ลดลง ดังนั้นอาจกล่าวได้ว่า LAAO จากพิษงูจงอางมีศักยภาพในการต้านมะเร็งผิวหนัง ซึ่งอาจพัฒนาต่อเพื่อใช้เป็นสารยับยั้งเซลล์มะเร็งผิวหนังได้

คำสำคัญ: L-amino acid oxidase, งูจงอาง, การตายแบบ apoptosis, เซลล์มะเร็งผิวหนัง

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Cytotoxic L-Amino Acid Oxidase Isolated from *Ophiophagus hannah* Induced Apoptosis in Human Melanoma Cell Lines

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Abstract

L-amino acid oxidase (LAAO) is one of the components in snake venom that is toxic to cells. There are few studies of anti-melanoma in Thailand. The King cobra, *Ophiophagus hannah*, is a large and poisonous snake with much venom. Therefore, this study aimed to investigate the cytotoxic potential of LAAO isolated from *O. hannah* venom in normal human skin fibroblast and human melanoma cell lines. LAAO was isolated by chromatographic procedure based on size exclusion, anion exchanger, and immuno-affinity. MTT assay showed that at 48 h *O. hannah* LAAO (OH-LAAO) exposure, cell death was rarely found in normal skin fibroblast, whereas approximately 27% death of melanoma cells was exhibited. Phosphatidyl serine externalization in apoptosis was detected by flow cytometry, which determined the apoptotic cell death induced by OH-LAAO in both cell types. This cell death involved reactive oxygen species, H_2O_2, generated from the OH-LAAO reaction. The pretreated catalase enzyme could remarkably prevent cell death induced by OH-LAAO. The cytochrome c release into the cytosol and the reduction of Bcl-2 expression indicated that the mitochondrial pathway was considered in the process of apoptosis induced by OH-LAAO. These results provided evidence of OH-LAAO's potential to be a further therapeutic agent for human melanoma.

Keywords: L-amino acid oxidase, *Ophiophagus hannah*, apoptosis, human melanoma

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Received: 26 January 2023 Revised: 19 June 2023 Accepted: 18 July 2023

Introduction

L-amino acid oxidases (LAAOs) are commonly found in the venom of snakes. The first isolation and characterization of snake LAAO have been reported in 1950.[1] The LAAOs were purified from various snake venoms: *Ophiophagus hannah,*[2] *Naja kaouthia*,[3] *Bothrops jararacussu*,[4] *Trimeresurus flavoviridis,*[5] and *Agkistrodon actus.*[6] *O. hannah* is widely distributed in Southeast Asia.[7] *O. hannah* venoms typically contain neurotoxins, cytotoxins, and enzymes, of which LAAO is notable. The LAAO is a flavin adenine dinucleotide (FAD) enzyme that contributes to the yellow appearance of the snake venom.[8] LAAO can catalyze the stereospecific oxidative deamination of L-amino acid into the corresponding alpha-keto acid, hydrogen peroxide (H_2O_2), and ammonia.[9]

LAAOs are multifunctional enzymes with the ability to produce reactive oxygen species (ROS), which participate in cell metabolism and possess protective effects.[10] LAAOs are glycoproteins with a molecular mass of 55-66 kDa (monomeric form).[11] LAAOs from snake venom have biological activities, including apoptosis-inducing activity on mammalian cells. The *in vitro* study reported that LAAO-generated H_2O_2 could induce oxidative stress in cancer cells, and lead to changes in cell morphology.[12] The H_2O_2 generation could be the major mechanism of cytotoxicity induced by LAAO, and many reports revealed that apoptosis induction by LAAO was associated with the killed cancer cells, including breast cancer,[13] lung adenocarcinoma,[14] and cervical cancer.[6] Generally, the induction of apoptosis can occur through extrinsic and intrinsic pathways. In the intrinsic pathway, the mitochondria play an important role in regulating cell death, which is mediated by regulating Bcl-2 family protein members. Excessive ROS generation will disrupt mitochondria permeability transition and release cytochrome c into cytosol, resulting in the activation of the execution caspases and the subsequent characteristics of apoptosis.[15]

This study was focused on snake LAAO and its effects on melanoma with the specific aims of 1) screening the LAAO activity of seven important Thai snake venoms, 2) purifying the high-activity LAAO from the selected snake venom, and 3) investigating the anti-melanoma potential of that LAAO. After the activity screening and the LAAO purification, the apoptosis induced by LAAO in normal human skin fibroblast and human melanoma cell lines was examined, including cell viability, phosphatidyl serine externalization, and H_2O_2 generation involving cell death both with and without catalase pretreatment. Finally, the appearance of cytochrome c in the mitochondria and the cytosol and Bcl-2 expression was also investigated.

Materials and Methods

Snake venoms

Seven pooled snake venoms (freeze-dried); *Daboia siamensis, Trimeresurus albolabris, Calloselasma rhodostoma, Naja kaouthia, Ophiophagus hannah, Bungarus fasciatus,* and *Bungarus candidus,* were

obtained from the snake farm, Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand.

Cell culture

The human melanoma cell line (SK-MEL-28, ATCC HTB-72) and normal human skin fibroblast cell line (CRL-1474, ATCC CCD-25Sk) were cultured in Dulbecco's Modified Eagle Medium (DMEM), Thermo Fisher Scientific, USA, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2.

L-amino acid oxidases screening activity

The LAAO activities of seven crude snake venoms *Daboia siamensis, Trimeresurus albolabris, Calloselasma rhodostoma, Naja kaouthia, Ophiophagus hannah, Bungarus fasciatus,* and *Bungarus candidus* were assayed by measuring the initial rate of H_2O_2 with a coupled peroxidase/dye assay.[16]

L-amino acid oxidase purification

Crude *O. hannah* venom (200 mg) was purified by ÄKTA pure machine (GE Healthcare Life Sciences, Germany) and applied to the Sephadex G-75 column, the Resource Q column, and the HiTrap™ Heparin affinity chromatography column (GE Healthcare Life Sciences, Germany). The fractions that showed LAAO activities were pooled and subsequently used for characterization and confirmation.

Determination of N-terminal amino acid sequence

The peptides samples were analyzed by electrospray ionization mass spectrometry using the Shimadzu Prominence nano HPLC system (Shimadzu) coupled to a 5600 Triple TOF mass spectrometer (Sciex, MA, USA). Tryptic peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.5 μm (Agilent Technologies), and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analyzed to identify proteins of interest using Mascot sequence matching software (Matrix Science, MA, USA) with the UniProt database.

OH-LAAO cytotoxicity determination by MTT assay

SK-MEL-28 and CRL-1474 cell lines were seeded on a 96-well plates at an approximate initial density of 2×10^4 cells/well and then treated with 25 μL of OH-LAAO at various concentrations. After 24, 48, and 72 h, MTT (5 mg/mL) was added to each well and incubated for 3 h. The medium was removed, then formazan crystals were dissolved by DMSO. The absorbance was read at 540 nm and results were expressed as a percentage of cell survival. All assays were done in triplicate and three independent experiments (n = 9). To investigate the cell death involving H_2O_2 generation, the cells were pretreated with 0.4 mg/mL catalase enzyme for 30 minutes, after that, the treatment with or without OH-LAAO for 24, 48, and 72 h was done. The cell survival was interpreted by MTT assay.

Determination of hydrogen peroxide (H_2O_2) production

The cell death of SK-MEL-28 and CRL-1474 induced by OH-LAAO was done and the culture media at 24, 48, and 72 h LAAO exposure were collected. The H_2O_2 generation in culture media was detected by the ™Hydrogen Peroxide Assay Kit (Abbkine Scientific, China).

Determination of cytochrome c and Bcl-2 expression by Western blot analysis

The OH-LAAO treated SK-MEL-28 and CRL-1474 cells at 24, 48, and 72 h were extracted in cold extraction buffer (20 mM Hepes-KOH, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.1 mM PMSF, pH 7.5). The resuspended cells was homogenized, then centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatants were collected and centrifuged at 13,000 rpm for 1 h at 4 °C. The supernatant from this step was used as cytosolic fractions. As the mitochondrial fractions, the pellets were resuspended with the mitochondrial extraction buffer (10 mM Tris-HCl pH 6.7, 0.15 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, 1 mM DTT, protease inhibitors) and vortex mixed for 10 seconds. The cytochrome c was determined both in the cytosolic and mitochondrial fractions. For Bcl-2 protein extraction, the OH-LAAO treated SK-MEL-28 and CRL-1474 cells at 24, 48, and 72 h were extracted by NP40 Cell Lysis buffer (Invitrogen, USA). Equal amounts of protein samples at 7 µg per well, measured by Qubit Protein Assay Kit, (Invitrogen, USA), were run in SDS-PAGE and transferred onto the blotting membrane. Then, the primary antibody (Abcam, USA) for Bcl-2, cytochrome c, or GAPDH, was added and incubated overnight at 4°C. The enzyme-conjugated secondary antibody (Sigma, USA) was added and incubated for 1 h at room temperature. The membrane was washed, and the substrate solution, 4-Chloro-1-naphthol (Sigma, USA) with H_2O_2, was poured on the blotting membrane to develop the blotting target bands. The density of each protein band from the western blot was quantified by UN-SCAN-IT gel version 7.1 software, USA, and normalized with GAPDH band density. The results were expressed in the relative optical density unit.

Apoptosis observation by flow cytometry

SK-MEL-28 and CRL-1474 cell lines were treated with OH-LAAO at the concentration of 6.25 µg/mL for 24, 48 and 72 h at cell density of 1×10^6 cells/mL. Cell samples were dual stained with fluorochrome-conjugated Annexin V and propidium iodide solution (BioLegend, USA). The samples were analyzed using FACSCalibur flow cytometry with CellQuest Pro software (Becton Dickinson, USA). This assay discriminates viable cells (annexin V-/PI-) from cells in early apoptosis (annexin V+/PI-), cells in late apoptosis (annexin V+/PI+), and cells undergoing necrosis (annexin V-/PI+). A total of 30,000 cells were acquired for each sample.

Statistical analysis

Statistical significance was determined by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons tests: PRIMER of Biostatistics software analysis. The differences were considered significant at $p < 0.05$.

Results

L-amino acid oxidases screening activity

The results of LAAO activities screening in seven crude snake venom samples found that *T. albolabris*, *O. hannah*, and *C. rhodostoma* possessed the top three of the highest LAAO activity with 2.66, 2.40, and 2.36 unit/mg, respectively, while *D. siamensis* gave the result of the lowest with 0.03 unit/mg. Because of sufficient venom supply and stable activity when it was stored in a freezer, *O. hannah* was selected for LAAO purification.

***O. hannah* L-amino acid oxidase purification**

Crude *O. hannah* venom was purified in three-step by size exclusion (Sephadex G-75 column), ion exchange (Resource Q column), and affinity chromatography (HiTrap™ Heparin column), respectively. Only a single peak (the box, Fig. 1A) represented the clear purity of OH-LAAO without other contaminated protein peaks. This OH-LAAO peak was kept and confirmed by SDS-PAGE. LAAO obtained from *O. hannah* showed a single protein band with around 56-57 kDa (Fig. 1B).

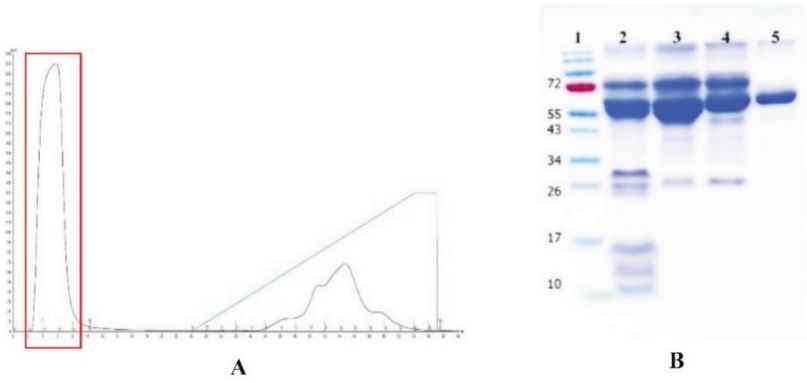

Fig. 1 OH-LAAO chromatogram and fractionated protein bands of *O. hannah* in SDS-PAGE analysis after purification through the HiTrap™ Heparin column using ÄKTA pure chromatography systems. The single OH-LAAO peak after being purified by HiTrap™ Heparin column (A), and the three-step chromatographic purification was run by SDS-PAGE (B), Lane 1; Protein Ladder Marker (PageRuler Prestained Protein Ladder, Thermo Fisher Scientific, USA.), lane 2; crude *O. hannah* venom, lane 3; *O. hannah* venom after purified by the Superdex G-75, lane 4; *O. hannah* venom after purified by the Resource Q column, and lane 5; the LAAO of *O. hannah* after purified by the HiTrap™ Heparin column.

Table 1 The purification results of OH-LAAO.

Step	Protein (mg)	Specific activity (U/mg)	Total activity	Purification (fold)	Yield (%)
1. Crude venom	200.00	2.40	480.00	1.00	100.00
2. Sephadex G-75 column	42.50	11.17	474.73	4.60	98.89
3. Resource Q column	21.50	15.99	343.79	6.70	71.60
4. HiTrap™ Heparin column	13.50	21.72	293.22	9.00	61.10

The data in Table 1 exhibited that crude *O. hannah* venom before purification gave the result of specific LAAO activity at 2.40 U/mg. After three steps of purification, the OH-LAAO showed higher activity at 21.72 U/mg with the fold purification of 9.00 and 61.10% yield. The OH-LAAO obtained by this purification was 13.50 mg protein, which was 6.75%, calculated from 200 mg crude *O. hannah* venom.

Determination of N-terminal amino acid sequences

The purified OH-LAAO product resulted in corresponding to OH-LAAO with 52% coverage using Mascot sequence matching software (Matrix Science, MA, USA) with the UniProt database (Fig. 2).

1 MNDFLLLLLV LFLGVPRSEN HVINLEECFQ EPEYENWLAT ASHGLTKTLN
51 PKKIVIVGAG ISGLTAAKLF REAGHEVVIL EASDRVGGRI KTHREDGWYV
101 DVGPMRVPQT HRIVREYIKK FNISLNPFRQ TDENAWYLIK HVRQKMSANN
151 PENFGYQLNP NERGKSASQL FDETLDKVTD DCTLQKEKYD SFSTKEYLIK
201 EGKLSTGAVE MIGDFLNEEA GFHNSFLISV MDHFLFLNNS FDEITGGFDQ
251 LPERFFKDMD SIVHLNSTVE KIVHINNKVT VFYEGLSTNM RLVADYVLIT
301 ATARATRLIK FVPPLSIPKT RALRSLIYAS ATKIILVCTD KFWEKDGIHG
351 GRSITDLPSR VIYYPNHDFT NGIGVLLASY TWYSDSEFYT TLSDEKCVDV
401 VMDDLVEIHN VSKDYLKSVC GKHVVQKWAL DQYSMGAFST YTPYQITHYS
451 QMLAQNEGRI YFAGEYTAHP HGWIETSMKS AIREAINIHN A

Fig. 2 The matching result of the OH-LAAO sequence (bold letters) with the OH-LAAO data on the UniProt database.

OH-LAAO cytotoxicity in CRL-1474 and SK-MEL-28 cell lines

To investigate the anti-cancer potential of LAAO purified from the *O. hannah* venom, the cytotoxic activity in normal human skin cells (CRL-1474) and melanoma cells (SK-MEL-28) were investigated. The results of this experiment were described in Table 2.

From Table 2, OH-LAAO at the concentration of 6.25 μg/mL showed 100% cell survival of CRL-1474 at 24 and 48 h LAAO exposure and slightly reduced the survival to

Table 2 The cell survival of CRL-1474 and SK-MEL-28 after treatment with OH-LAAO.

% Cell survival	h	OH-LAAO concentration (μg/ml)					
		1.56	3.12	6.25	12.5	25	50
CRL-1474	24	100.00±11.66	100.00±11.76	100.00±14.43	77.70±18.67	47.30±7.83	25.35±6.14
	48	100.00±7.02	100.00±7.84	100.00±12.12	56.56±14.17	35.07±8.19	17.32±1.74
	72	100.00±8.76	100.00±3.37	85.34±4.55[a]	38.65±5.70	18.13±2.95	18.02±2.97
SK-MEL-28	24	100.00±5.10	100.00±6.79	86.87±18.16[*]	72.92±19.65	46.39±12.45	43.93±8.10
	48	99.98±4.10	95.76±10.03	73.30±14.23[*]	54.68±11.16	34.50±6.87	22.18±4.02
	72	89.30±5.47	74.28±11.75[c]	68.58±8.13[*, b]	46.78±10.87	30.26±5.86	20.76±3.69

* = significantly different when compared to % cell survival of CRL-1474 at the same time; a = significantly different when compared to % cell survival of CRL-1474 at 24 and 48 h; b = significantly different when compared to % cell survival of SK-MEL-28 at 24 h; c = significantly different when compared to % cell survival of SK-MEL-28 at 24 and 48 h; Cell survival was investigated by MTT assay. Data were presented as mean ±S.D. Statistical significance was performed by ANOVA and Bonferroni's multiple comparisons tests with PRIMER of Biostatistics software. $p < 0.05$ was considered to be statistically significant.

85% at 72 h. Whereas OH-LAAO decreased the cell survival of SK-MEL-28 to 87% even at 24 h exposure and significantly decreased the survival to 69% at 72 h. The lower concentration than 6.25 μg/mL was not potent enough to kill SK-MEL-28 cells at 24 h, although the percentage of SK-MEL-28 survival was significantly lower when the LAAO exposure was longer. For the upper concentration over 6.25 μg/mL, OH-LAAO gave the results of less survival in both cells, about less than 50% survival. Therefore, the cells were treated with 6.25 μg/mL.

Antioxidants such as catalase and glutathione (GSH) can scavenge excessive ROS, such as H_2O_2, and protect cells from damage by oxidative stress. Therefore, OH-LAAO was further investigated for the effects of the pretreatment with 0.4 mg/mL catalase enzyme. The results of the MTT assay were shown in Table 3.

Table 3 The percentage of SK-MEL-28 cell survival with or without catalase enzyme condition.

OH-LAAO (6.25 μg/mL)	% SK-MEL-28 Survival		
	24 h	48 h	72 h
With catalase	100.00±4.56*	89.07±12.16*	100.00±10.86*
Without catalase	86.87±15.16	73.30±14.23	68.58±8.13
Catalase enzyme alone	97.69±6.84	97.28±3.13	100.00±10.77

* = significantly different when compared to % SK-MEL-28 survival without catalase at the same time
Cell survival was investigated by MTT assay. Data were presented as mean±S.D. Statistical significance was performed by ANOVA and Bonferroni's multiple comparisons tests with PRIMER of Biostatistics software. A $p < 0.05$ was considered to be statistically significant.

In this experiment, SK-MEL-28 cells pretreated with catalase enzyme continuously survived from 24 to 72 h despite exposure to OH-LAAO, the results of CRL-1474 were the same (data not shown). Catalase enzyme alone did not affect cell survival, but catalase would be able to scavenge H_2O_2 produced by OH-LAAO reaction and gave the consequence of the survival rate increase in OH-LAAO treated cells.

Determination of Hydrogen peroxide production

Several LAAOs can promote apoptosis of tumor cells mediated by the release of H_2O_2, resulting in oxidative stress. To confirm the appearance of H_2O_2 after OH-LAAO exposure, the amounts of H_2O_2 in the culture media of the cells treated with OH-LAAO were measured.

Table 4 Generation of H_2O_2 in culture media of CRL-1474 and SK-MEL-28 treated with OH-LAAO.

Amount of H_2O_2 (μM)	Without OH-LAAO			With OH-LAAO		
	24 h	48 h	72 h	24 h	48 h	72 h
CRL-1474 media	13.31±0.21	13.36±0.37	13.67±0.30	377.34±1.80	328.49±1.65	291.42±1.10
SK-MEL-28 media	13.63±0.34	13.70±0.32	13.70±0.30	362.52±1.33*	334.78±1.36*	324.21±1.37*

* = significantly different when compared to the amount of H_2O_2 in CRL-1474 treated with OH-LAAO at the same time
Data were presented as mean±S.D. Statistical significance was performed by ANOVA and Bonferroni's multiple comparisons tests with PRIMER of Biostatistics software. A $p < 0.05$ was considered to be statistically significant.

The results shown in Table 4 confirmed that H_2O_2 could be produced in cell culture media by the purified OH-LAAO. The H_2O_2 in the culture media of both SK-MEL-28 and CRL-1474 cell lines remarkably appeared when the cells were treated with OH-LAAO. The amount of generated H_2O_2 in each cell type was gradually reduced with a significant difference from 24 to 72 h exposure. However, the amount of H_2O_2 that occurred in OH-LAAO-treated SK-MEL-28 cells was significantly higher than that of the treated CRL-1474 cells. There was L-amino acid in the culture media as the substrate of OH-LAAO, so H_2O_2 could be generated by the OH-LAAO reaction, which was toxic to cells.

Determination of cytochrome c and Bcl-2 expression by Western blot analysis

LAAOs can lead to oxidative stress formation in cells resulting from elevated ROS levels and induce the intrinsic apoptotic pathways (mitochondria pathway) by upregulating pro-apoptotic proteins, including cytochrome c, and decreasing apoptotic regulatory proteins, such as Bcl-2, in cells. Western blot analysis was done to check the actual occurrence expression of cytochrome c and Bcl-2 protein (Fig. 3A).

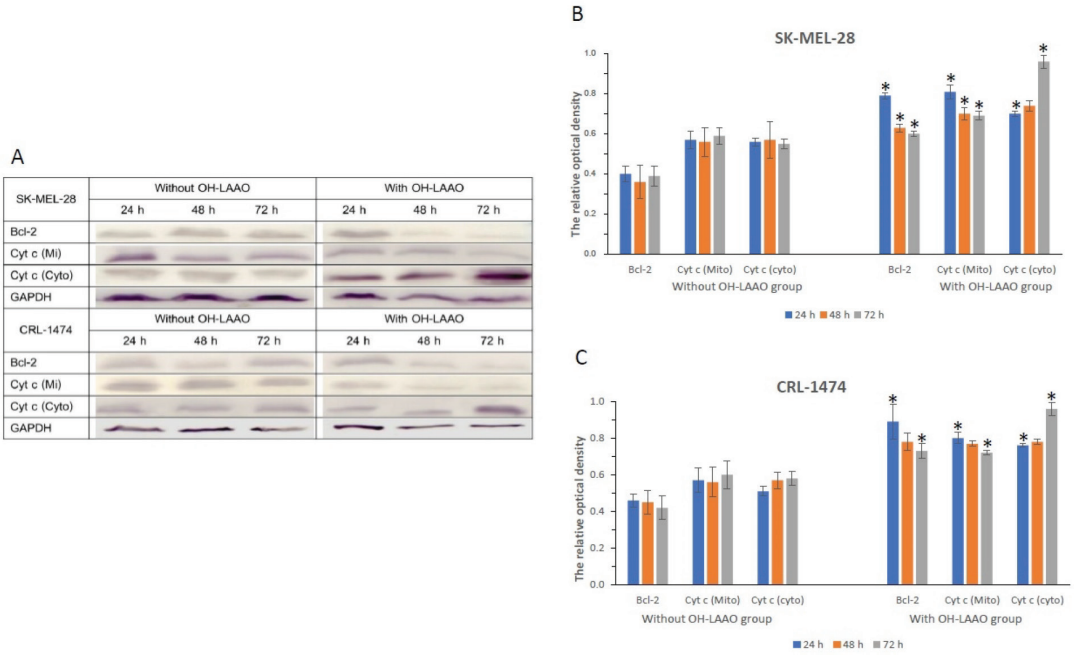

Fig. 3 The expression of Bcl-2, mitochondrial, and cytosolic cytochrome C in SK-MEL-28 and CRL-1474 cells after treatment with and without OH-LAAO. A, western blots showing the expression of Bcl-2, mitochondrial, and cytosolic cytochrome C of SK-MEL-28 and CRL-1474 cells treated by OH-LAAO for 72 h. B-C, band densities neutralized with GAPDH band densities, were shown.

Apoptosis induced by OH-LAAO in SK-MEL-28 and CRL-1474 cell lines

LAAOs have been studied and they were shown to induce cytotoxicity and apoptosis in tumor cells. PI-Annexin V detection with flow cytometry was investigated to ensure that apoptosis occurred in SK-MEL-28 cells after treated OH-LAAO, 6.25 µg/mL. The results are shown in Table 5.

OH-LAAO at 6.25 µg/mL concentration induced apoptosis more than necrosis in both cell lines. Excluding the viable cells, apoptosis including early and late apoptosis found in the with-OH-LAAO groups was significantly higher than those of the without-OH-LAAO groups in both cell types. Whereas the percentage of necrosis was relatively low and was not significant even though each cell type was treated with OH-LAAO. Total apoptosis exhibited in each cell line was significantly high after 48 h after OH-LAAO exposure.

Discussion

The important venomous snakes in Thailand would be *D. siamensis*, *T. albolabris*, *C. rhodostoma*, *N. kaouthia*, *O. hannah*, *B. fasciatus*, and *B. candidus*, which could be classified into hemotoxic and neurotoxic types. However, their LAAO contributing to the

Table 5 The total apoptosis and necrosis percentage of SK-MEL-28 cells cultured with/without OH-LAAO from 24 h to 72 h.

SK-MEL-28 (without OH-LAAO)			SK-MEL-28 (with OH-LAAO)		
%Total apoptosis					
24 h	48 h	72 h	24 h	48 h	72 h
12.71±0.25	15.83±1.33	19.30±1.57[a, b]	20.12±1.72*	25.59±1.00[*, c]	35.07±1.68[*, c, d]
%Necrosis					
24 h	48 h	72 h	24 h	48 h	72 h
0.85±0.47	1.29±0.67	1.34±0.65	0.74±0.34	0.89±0.71	1.15±1.05

CRL-1474 (without OH-LAAO)			CRL-1474 (with OH-LAAO)		
%Total apoptosis					
24 h	48 h	72 h	24 h	48 h	72 h
4.21±2.34	9.79±1.74	10.09±2.90	17.39±1.85*	27.40±2.18[*, c]	32.83±2.73[*, c]
%Necrosis					
24 h	48 h	72 h	24 h	48 h	72 h
0.53±0.25	0.44±0.22	0.52±1.79	1.09±0.86	0.25±0.13	0.37±0.32

* = significantly different when compared to % total apoptosis of the same cell type without OH-LAAO treatment at the same time;
a = significantly different when compared to % total apoptosis of the same cell type without OH-LAAO treatment at 24 h;
b = significantly different when compared to % total apoptosis of the same cell type without OH-LAAO treatment at 48 h;
c = significantly different when compared to % total apoptosis of the same cell type with OH-LAAO treatment at 24 h;
d = significantly different when compared to % total apoptosis of the same cell type with OH-LAAO treatment at 48 h;
Data were presented as mean±S.D. Statistical significance was performed by ANOVA and Bonferroni's multiple comparisons tests with PRIMER of Biostatistics software. A $p < 0.05$ was considered to be statistically significant.

yellow appearance displayed individual activity depending on each species. Because of large snakes with much venom and their high LAAO activity, *O. hannah* and *C. Rhodostoma,* were considered. Owing to the observable instability of *C. rhodostoma,* LAAO activity was not found after freezing for a week; however, the study of Lee *et al.* (2013)[16] reported uncommon thermal stability and no inactivation by freezing. *O. hannah* venom was chosen for further LAAO purification.

LAAO purification was done in three steps. The last step of purification was done using the HiTrap™ Heparin column, negatively charged polydisperse linear polysaccharides which could bind a wide range of biomolecules including LAAO enzymes. Consequently, the OH-LAAO product had high purity and gave the single band with around 56-57 kDa (Fig 1(B), lane 5). The result of N-terminal amino acid sequencing with 52% coverage exhibited that this purified protein was LAAO,

with a molecular weight of 55.941 kDa and pI of 5.99.

The results of the MTT assay determined the cytotoxic activity of OH-LAAO which caused cell death in a concentration- and time-dependent manner. The suitable concentration of OH-LAAO in this study should be 6.25 µg/mL, or 0.156 µg/2 x10^4 cells/well, which was less harmful to normal human skin fibroblasts but affected human melanoma with approximately 27% cell death (Table 2). Based on these results of direct toxicity tests on cells, OH-LAAO at 0.156 µg might be the starting dose for therapeutic tests in an animal model which might be the melanoma-implanted nude mice injected intradermally with OH-LAAO. Due to the narrow effective dose (>0.078 to <0.312 µg), tested OH-LAAO doses must be carefully adjusted. Nevertheless, the tested doses might be higher if the route of administration and cancer types are different. For example, 20 µg of OH-LAAO was used for intraperitoneal injection to suppress human prostate adenocarcinoma (PC-3) solid tumor growth in implanted nude mice.[17] OH-LAAO might be used in a short time for cancer therapy. Therefore, the observation time should be carefully done within 48 h, because the normal cell might be destroyed if the OH-LAAO exposure was longer. However, the optimum dose and time for the reduction of tumor size by OH-LAAO should be further investigated. As a scavenger of H_2O_2, the pretreatment with catalase enzyme could significantly increase % cell survival despite exposure to OH-LAAO (Table 3). This showed that the cell death induced by OH-LAAO involved the H_2O_2 production by this enzyme reaction. ROS peroxides are known to regulate cell death in a variety of cell types. High levels of ROS can lead to lipid peroxidation,[18] cellular membrane damage,[19] inactivation of caspase enzymes, and necrotic cell death.[20] LAAO reacts with L-amino acid to generate H_2O_2 which induced oxidative stress and then leads to the death of cells[21] Therefore, the H_2O_2 in the culture media of SK-MEL-28 and CRL-1474 treated with OH-LAAO was measured. The results of both with and without OH-LAAO in these two cell types presented that the amount of generated H_2O_2 was in the low range which could induce apoptosis. If it could induce predominant necrosis, the amount of generated H_2O_2 should be higher (1-10 mM).[22] Moreover, the amount of H_2O_2 that occurred in OH-LAAO-treated SK-MEL-28 cells was significantly higher than those of CRL-1474, which correlated to the higher cell death of SK-MEL-28 exposed to OH-LAAO (Table 4). The significant gradual reduction of H_2O_2 generation in both cell types from 24 to 72 h OH-LAAO exposure (Table 4) might be a result of the limitations of the LAAO substrate resource (L-amino acid) in the cell culture media.

The Western blots demonstrated the expressed proteins in the apoptosis pathway after treatment with OH-LAAO. The mitochondria and cytosol were extracted to detect the cytochrome c expression levels. The cytochrome c expression in mitochondria was decreased after longer exposure to OH-LAAO,

corresponding to the increased cytochrome c protein expression in the cytosol. Cytosolic cytochrome c can activate caspases in the apoptosome and finally lead to the activation of caspase 3, subsequently leading to apoptosis.[23] These suggest that OH-LAAO could induce apoptosis by cytochrome c increasing in the cytosol, however, the exact mechanism is still unclear.[8, 24] Bcl-2 is one of the anti-apoptotic members[25] and an apoptosis suppressor gene,[26] and the overexpression of Bcl-2 protein in cancer cells may block or delay the beginning of apoptosis.[27] ROS acts to down-regulate endogenous Bcl-2 levels within cells because levels of Bcl-2 within cells are critical to antiapoptotic activity.[28] The Bcl-2 decreasing can be a mechanism to sensitize cells to apoptosis. In addition, the preliminary observation of apoptosis enzymes expressed in SK-MEL-28 and CRL-1474 cells induced by OH-LAAO showed a higher level of caspases-3 and -9 when compared to those of untreated SK-MEL-28 and CRL-1474 cells, using Human Caspase-3 and -9 ELISA Kits (CUSABIO, USA) (the data not shown). However, the expression of caspase-3 and -9, in addition to the others related to apoptosis, should be finely confirmed in a further study to indicate the evident apoptosis pathway of OH-LAAO. Caspase-3 and 9 were triggered by cytochrome c in the cytosol to activate DNA degradation in the apoptotic pathway.

The dual staining of annexin V/PI binding assay is a powerful tool for the quantification of apoptosis and for distinguishing between apoptotic and necrotic cells. It is widely used as a signature of apoptotic cell death.[29] In results of flow cytometry analysis exhibited the binding of annexin V, suggesting that phosphatidyl serine externalization during apoptosis occurred. OH-LAAO had the capability of inducing apoptosis in a time-dependent manner which significantly occurred at 24 h compared to without OH-LAAO treatment and significantly increased since 48 h after OH-LAAO exposure. The cell death induced by OH-LAAO in normal human skin fibroblast cell lines (CRL-1474) and melanoma cells (SK-MEL-28) was mostly apoptosis. Necrosis induced by OH-LAAO was very slight; even though, the exposure time was 72 h long (Table 5).

In conclusion, Thailand's seven significant venomous snakes possessed individual LAAO activity. With only the single protein band purity, the LAAO obtained from *O. hannah* venom by this three-step chromatographic purification revealed cytotoxic activity in both normal human skin fibroblasts, CRL-1474, and human melanoma, SK-MEL-28. Cell death was rarely found in CRL-1474, whereas approximately 27% of cell death was exhibited in SK-MEL-28 when both cell types were treated with 0.156 µg of OH-LAAO for 48 h. The majority of cell death induced by OH-LAAO was apoptosis. The mechanism of OH-LAAO involves H_2O_2 generation and the mitochondrial apoptotic pathway. The optimum dose and time, the pharmacokinetics of OH-LAAO, and its cumulative dose must be considered for further studies. This evidence presented the potential

of OH-LAAO as a candidate for the further study of therapeutic agents for human melanoma treatment.

Acknowledgements

This work was supported by a grant (project No. QSMI6406) from The Scientific Committee of the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand.

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