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Methylantcinate A induces tumor specific growth inhibition in oral cancer cells via Bax-mediated mitochondrial apoptotic pathway

Wan-Chi Tsai [a,b], Yerra Koteswara Rao [c], Shih-Shen Lin [d], Ming-Yung Chou [d], Yi-Ting Shen [a], Chih-Hao Wu [a], Madamanchi Geethangili [c], Chi-Chiang Yang [a,b,*], Yew-Min Tzeng [c,*]

[a] School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, ROC
[b] Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan, ROC
[c] Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Wufeng, Taiwan, ROC
[d] School of Dentistry, Chung Shan Medical University, Taichung, Taiwan, ROC

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ABSTRACT

An ergostane type triterpenoid methylantcinate A (MAA) isolated from the fruiting bodies of *Antrodia camphorata* inhibited the growth of oral cancer cell lines OEC-M1 and OC-2 in a dose-dependent manner, without cytotoxic to normal oral gingival fibroblast cells. The major mechanism of growth inhibition was apoptosis induction, as shown by flow cytometric analysis of annexin V-FITC and propidium iodide staining, caspase-3 activation and DNA fragmentation. The increased expression of pro-apoptotic Bax, poly-(ADP-ribose) polymerase cleavage, and activated caspase-3 and decreased expression of anti-apoptotic Bcl-2 and Bcl-xL were also observed. These results provide the first evidence that the anti-oral cancer effects of MAA may involve a mechanism through the mitochondrial dependent pathway. Thus, results reported here may offer further impulse to the development of MAA analogues as potential chemotherapeutic targets for oral cancer complications.

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, and accounts for approximately 4% of all carcinomas in men and 2% in women worldwide, with geographical variation in frequency.[1] Particularly, OSCC is the fourth leading cause of male cancer mortality in Taiwan, and the risk factors include betel quid chewing, cigarette smoking, and alcohol drinking.[2,3] Despite multidisciplinary treatment with surgery, chemotherapy, and radiation, the overall survival rate has not improved significantly in patients with oral cancer.[4] In addition, current chemotherapeutic agents have limited efficacy in oral cancer, and recently resistance to oral cancer drugs has been observed.[4] Therefore, the development of potential anti-oral cancer agents which are less toxic to the normal cell population has become necessary.

Approaches in the development of new anticancer therapeutics are focusing on the selective induction of tumor cell death through the activation of apoptotic pathways. Apoptosis is a highly controlled cellular process, that is, essential for the maintenance of normal tissue homeostasis and embryonic development. However, a failure to appropriately induce apoptosis often results in the accumulation of defective cells that are symptomatic of disease states such as cancer.[5] Therefore, molecules that selectively induce apoptosis in tumor cells are of considerable interest in the clinical management of cancer. The identification of several key protein targets that regulate apoptosis has resulted in the development of molecules that bind to one (or more) of these proteins and activate apoptotic pathways.[5] Models in vitro using human cancer cell lines are useful to study molecular targets against several tumors carcinogenesis, and provide important preliminary data to select compounds with potential anticancer properties for future study. Interest in the use of natural products has grown dramatically in the Western world. Recent estimates suggest an overall prevalence for herbal preparation use of 13–63% among patients with cancer.[6]

Antrodia camphorata (Niu-Chang-Chih or Zhan-Ku), Polyporaceae, is a medicinal mushroom that has been used for centuries in Taiwan for the prevention and treatment of various diseases including alcohol and drug intoxication, skin itching and, oral, and liver cancer.[7] The crude extracts of *A. camphorata* have a broad range of biological activities including anticancer in various cancer cell types.[8] Recently, studies were focused on identification of anticancer compounds, and evaluate their mechanism of action.[8] We have recently reported that triterpenoids isolated from the fruiting bodies of *A. camphorata* showed strong cytotoxic effects in various cancer cell types such as colon, liver, breast, and lung, and their apoptosis-inducing activities mediated by the mitochondrial dependent pathway.[9] Although, traditionally *A. camphorata* has been used for as a remedy for oral cancer, however, the

* Corresponding authors. Tel.: +886 4 24730022x11716; fax: +886 4 23767469 (C.-C.Y.); tel.: +886 4 23323000x4471; fax: +886 4 23395870 (Y.-M.T.).
E-mail addresses: cyang@csmu.edu.tw (C.-C. Yang), ymtzeng@cyut.edu.tw (Y.-M. Tzeng).

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identification of active compounds and their action mechanism against oral cancer has not been clearly elucidated. As part of our screening program for bioactive natural products include anticancer compounds,[10] here we report the isolation of another triterpenoid, methylantcinate A (MAA, Fig. 1) from *A. camphorata*, and its anti-proliferation and apoptosis activities in human OSCC.

The air-dried powder from the fruiting bodies of *A. camphorata* (101.9 g) was successively extracted (3 × 1000 ml) with *n*-hexane, chloroform, and methanol under reflux. After exhaustive extraction, the combined extracts were concentrated individually under reduced pressure to yield 3.02 g (2.96% based on dry weight) of *n*-hexane, 46.3 g (45.43%) of chloroform, and 2.7 g (2.64%) of methanol residues. Repeated silica gel column chromatography (5 × 90 cm) of the chloroform extract residue eluted with increasing polarity using mixtures of hexane/EtOAc. Following the TLC analysis, eluates of similar profiles were combined to give six fractions (F1–F6). Fraction F6 was purified by silica gel column chromatography using $CHCl_3$/MeOH from 100% $CHCl_3$ to 20% MeOH to yield MAA (Fig. 1). The structure of MAA was determined by ^1H and ^{13}C NMR spectroscopy and by comparison of the spectral data with those published values.[11]

The anti-proliferation effect of MAA was examined in human OSCC cell lines OC-2 and OEC-M1, obtained from adult male Taiwanese with a history of betel quid chewing, as well as the normal gingival fibroblast (GF) cells. Aforementioned three cell lines were treated with various concentrations of MAA for 24 h, and the viable cells were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay.[12] The MTT assay measures cell viability and proliferation based on the ability of the mitochondrial dehydrogenase enzymes to convert the yellow tetrazolium MTT to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. The amount of dye produced is proportional to the number of metabolically live cells. The results show that MAA exhibited a significant (p <0.01) and a dose-dependent inhibitory effect on OC-2 and OEC-M1 cells viability (Fig. 2). The concentration of MAA that results in a 50% reduction in absorbance (IC_{50}) compared with the control for OC-2 and OEC-M1 cells after 24 h of treatment were 37.4 μM and 24.5 μM, respectively. Although OEC-M1 and OC-2 were both established from primary tumors from adult male OSCC patients from Taiwan with a history of betel quid chewing,[13] however, the cytotoxic effects of MAA were different in these two cell lines. OEC-M1 was more sensitive to MAA than OC-2. Interestingly, the tested compound MAA could not affected the proliferation of normal GF cells at the maximum concentration tested (50 μM) suggesting a selective activity against tumor cells (Fig. 2).

Several additional methods were used to examine the mechanism of MAA-induced cell death. Apoptosis is accompanied by loss of organization in the plasma membrane, and a marker of this event is the translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer portion of the membrane.[14] Annexin V-FITC is a PS-binding protein that can be conjugated to fluorescent groups and used in FACS analysis to determine cell viability.

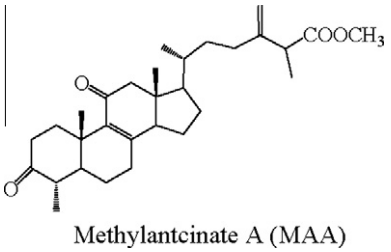

Figure 1. Chemical structure of methylantcinate A (MAA).

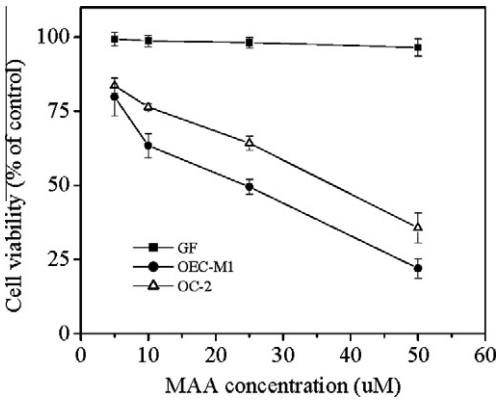

Figure 2. The anti-proliferation effect of MAA in oral cancer cell lines OC-2 and OEC-M1, and the normal oral fibroblast (GF) cells after 24 h of treatment. The p value was <0.01 and <0.05 in OEC-M1 and OC-2, respectively, when compared to DMSO control. There's no statistical significance between GF and DMSO control group.

Annexin V-FITC is typically used in conjunction with propidium iodide (PI), a fluorescent molecule that is impermeable to cells with intact membranes but permeable to dead cells. In this letter, FACS analysis of untreated OEC-M1 and OC-2 cells, the majority of cells appeared negative for staining with annexin V-FITC and PI, indicating that the cells were healthy (Fig. 3). Upon the treatment with MAA for 3 h, FACS analysis showed significant shifts in the cell population. The most significant new population of cells was positive for both annexin V-FITC and PI, indicating these cells had already died either by necrosis or apoptosis. An additional cell population that is more positive for annexin V-FITC than for PI was also observable (Fig. 3), indicating that these cells were undergoing apoptosis. Specifically, the percentage of healthy OEC-M1 cells positive for annexin V-FITC were decreased and, new apoptotic populations of cells increased from 0.69% to 59.73% and 62.33% with MAA treatment at 25 μM and 50 μM, respectively. Under the same conditions, OC-2 cells apoptotic populations were increased from 0.67% to 15.35% and 9.09% (Fig. 3).

The presence of additional apoptotic indicators were examined for both the cell lines under conditions where the cells were treated with MAA. During apoptosis, several caspase proteins are converted from the inactive (pro-form) to the active form. Caspase-3 is the major executioner caspase in the caspase cascade,[15] therefore following experiments were performed to characterize the role of activation of this protein in cell growth inhibition mediated by MAA. The caspase-3 activity was measured by flow cytometry following treatment with MAA using specific peptide substrate.[16] As shown in Figure 4A, MAA significantly increased the caspase-3 activity in OEC-M1 and OC-2 cells when added to culture medium for 3 h at 25 μM and 50 μM concentrations. Specifically, the caspase-3 activity increased from 52.34% to 66.86% and 87.07% in 25 μM and 50 μM of MAA treatment, respectively. Under the same conditions in OC-2 cells, caspase-3 activity increased from 48.99% to 59.19% and 69.06% (Fig. 4A). The ascent of caspase-3 activity in a time-dependent manner was also observed when OEC-M1 and OC-2 cells were treated with 50 μM of MAA for 3 h, 6 h, and 9 h (data not shown).

To further confirm the effect of MAA on induction of apoptosis, fragmentation of chromosomal DNA was analyzed (Fig. 4B) using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and flow cytometry.[16] The DNA fragmentation levels in OEC-M1 cells treated for 4 h with 25 μM and 50 μM of MAA increased from 8.15% to 56.5% and 57.8%, respectively. Under the same conditions, DNA fragmentation levels in OC-2 cells were increased from 28.6% to 41.07% and 52.55% (Fig. 4B).

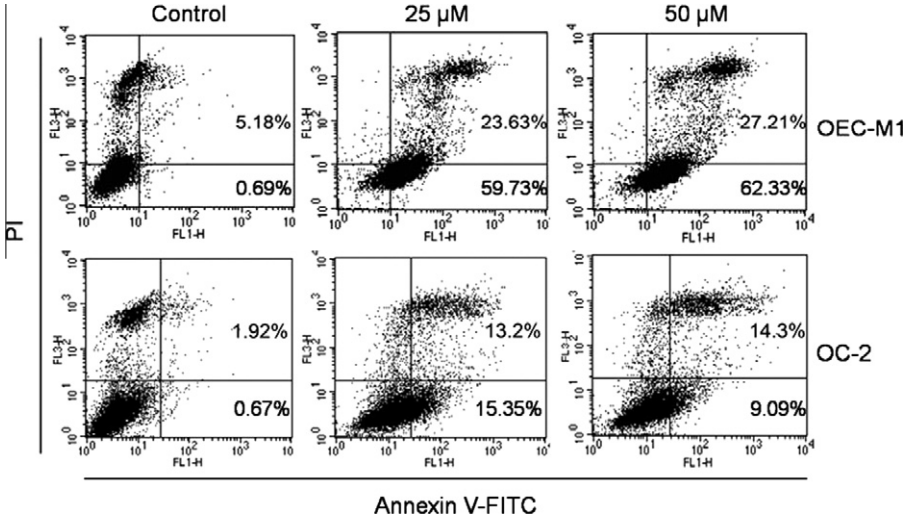

Figure 3. MAA-induced apoptosis. The tumor cell lines OEC-M1 and OC-2 were treated with 25 μM and 50 μM MAA or DMSO for 3 h, and then stained with annexin V-FITC and propidium iodide. Annexin V-FITC signal is shown on the *X* axis; propidium iodide (PI) signal is shown on the *Y* axis. Ten thousand events were collected for each sample. The percentage indicated the population of cells with annexin V-FITC/PI double positive signal.

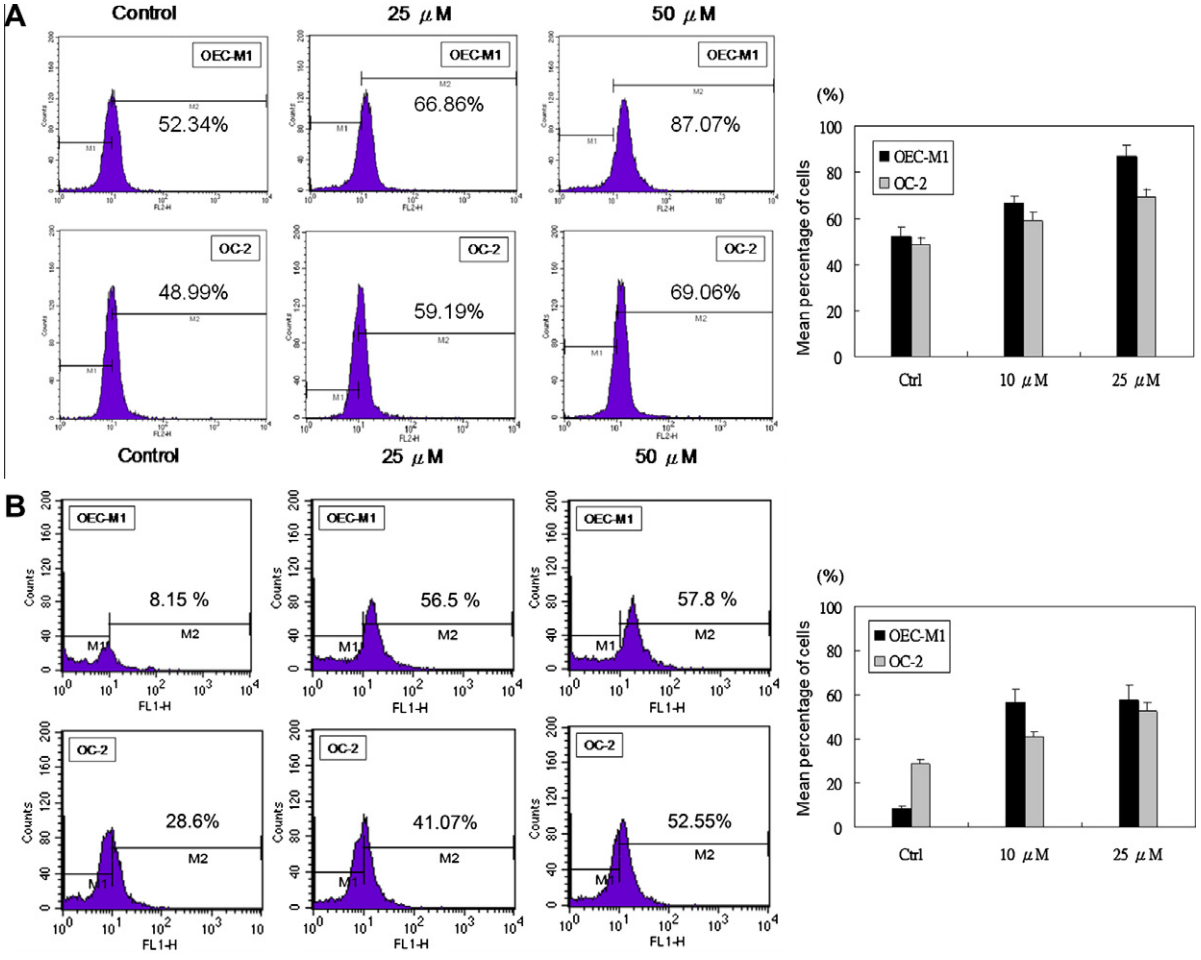

Figure 4. Effect of MAA on caspase-3 activity and DNA fragmentation in tumor cell lines OEC-M1 and OC-2. Cell lines were treated with 25 μM and 50 μM MAA or DMSO. (A) Flow cytometric analysis carried out after 3 h treatment to determine caspase-3 activity; (B) after 4 h treatment, cytoplasmic histone-associated DNA fragmentation was analyzed. The quantitative data are mean ± SD of three determinations.

To identify the molecular mechanism by which MAA induces apoptosis, we examined the levels of apoptosis regulatory proteins in MAA treated OEC-M1 cells using western blotting. Among the 11 caspases characterized in humans, caspase-3 is the main downstream effecter caspase that play essential roles in degrading the majority of key cellular components in apoptotic cells.[15] We

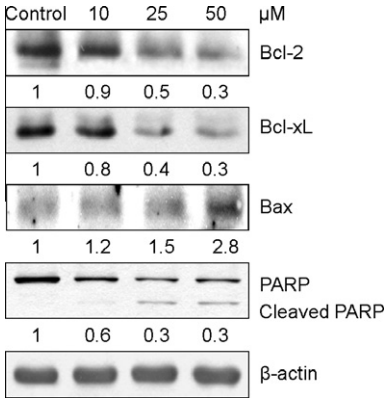

Figure 5. Effect of MAA on apoptosis regulatory proteins. OEC-M1 cells were treated with 10 μM, 25 μM, and 50 μM of MAA or DMSO for 6 h, total protein was isolated. Equal amount of cell lysates were analyzed for Bcl-2, Bcl-xL, Bax, and PARP protein expression by western blotting with corresponding antibodies. β-actin serves as the loading control. The values in fold denote the relative intensity of protein bands of MAA-treated samples to that of the respective DMSO vehicle–treated control after being normalized to the respective internal reference (β-actin). The values in the third row denote the relative fold of PARP but not cleaved PARP.

therefore investigated the possible involvement of caspase-3 in MAA-induced apoptosis. The activation of caspase-3 was monitored through detection of its proteolytic cleavage.[16] Treatment of OEC-M1 cells with MAA for 6 h promoted dose-dependent cleavage of poly (ADP-ribose) polymerase (PARP) from the full-length 116-kDa to an inactive 85-kDa form by activated caspases (Fig. 5) which is another indicator of apoptosis.[17] Many lines of evidence demonstrate that Bcl-2-related proteins play an important role in either inhibition or promotion of apoptosis.[18] The anti-apoptotic proteins Bcl-2 and Bcl-XL act to prevent permeabilization of the outer mitochondrial membrane by inhibiting the action of the pro-apoptotic protein Bax.[19] Our results from western blot analysis showed that the treatment of OEC-M1 cells with MAA for 6 h resulted, the increased expression of pro-apoptotic Bax protein and decreased expression of anti-apoptotic Bcl-2 and Bcl-xL proteins in a dose-dependent manner (Fig. 5). This finding suggested that the effect of the *Bax* gene product via the mitochondria might be responsible for the modulation of MAA-induced apoptosis in the OEC-M1 cells.[14] It is unclear at the present time whether MAA-induced apoptosis in OEC-M1 cells was reactive oxygen species (ROS) dependent and remain to be further elucidated. However, our recent results indicated that MAA-induced apoptosis in human liver cancer cells through ROS-dependent cofilin- and Bax-triggered mitochondrial pathway.[20] To our knowledge, this is the first letter on the anti-proliferation effect of MAA in oral cancer cells, and might exert pro-apoptotic effects through a Bax-mediated mitochondrial apoptotic pathway and caspase cascade in OEC-M1 cells.

In conclusion, this study provided the first evidence that methylantcinate A attenuates the growth of human oral cancer cells, triggering the Bax-mediated mitochondrial apoptotic pathway. Although the molecular mechanism by which apoptosis is induced by this natural compound remains to confirm, and of course further studies are needed, the results reported here may offer also a further impulse to the development of its analogues as potential chemotherapeutic targets for oral cancer complications.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.006.

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