Original Article
Int J Oral Biol 49:42-47, 2024
pISSN: 1226-7155 • eISSN: 2287-6618
https://doi.org/10.11620/IJOB.2024.49.2.42

International Journal of Oral Biology
IJOB
Check for updates

Antitumor effects of ophiopogonin D on oral squamous cell carcinoma

Nguyen Thi Kieu Trang[1,2], Vu Phuong Dong[1], and Hoon Yoo[1]*

[1]Department of Pharmacology and Dental Therapeutics, College of Dentistry, Chosun University, Gwangju 61452, Republic of Korea
[2]Department of Pharmacy, Thai Binh University of Medicine and Pharmacy, Thai Binh City 06000, Vietnam

Ophiopogonin D (OPD) is a steroidal glycoside derived from *Ophiopogon japonicus*, a traditional Chinese medicine with diverse biological activities, including antithrombosis, anti-inflammation, and antitussive effects. To investigate the cellular effects and mechanisms of OPD on oral squamous cell carcinoma, cell viability was explored, and the effects of OPD on cell cycle regulators, apoptotic marker proteins, and key proteins involved in metastasis and signaling pathways were examined by MTT assay and Western blotting in YD38 cells. OPD strongly inhibited cell proliferation and induced caspase-dependent apoptosis of YD38 cells by suppressing the cell cycle and activating caspase-3 and poly ADP ribose polymerase. Additionally, OPD suppressed the expression of vital proteins regulating metastasis and proliferation within the integrin/matrix metalloproteinases/FAK and AKT/PI3K/mTor pathways. Thus, OPD can be a potential treatment candidate for gingival cancer.

Keywords: Ophiopogonin D, Proliferation, Metastasis, Apoptosis, YD38 cells

Introduction

Oral cancers which affect the oral cavity and oropharynx represent a significant health concern. Particularly, in certain developing countries, they rank among the top three for cancer incidence [1]. Among oral malignancies, oral squamous cell carcinoma (OSCC) predominates, constituting roughly 90% of cases. Unfortunately, most OSCC diagnoses occur at advanced stages, resulting in the five-year disease-specific survival rate of only 50–60% [2]. The location of OSCC significantly impacts patients' quality of life, often impairing critical functions such as speech and taste [3]. Despite the advance in conventional treatments, including chemotherapy and radiotherapy, approximately one-third of patients experience recurrence and develop resistance to therapy [4,5]. Therefore, there is a pressing need for novel and optimized treatment approaches.

Ophiopogon japonicus, a traditional Chinese medicine, has drawn attention for its therapeutic properties in managing cardiovascular and inflammatory conditions [6–9]. Its beneficial effects on cardiovascular health include anti-ischemic and anti-arrhythmic activities, platelet aggregation inhibition, and microcirculation enhancement [10,11]. Ophiopogonin D (OPD), a steroidal glycoside derived from *O. japonicus*, also demonstrated diverse biological activities, including anti-thrombotic and anti-inflammatory effects, as well as antitussive properties [12–15]. However, its potential anticancer activity against OSCC remains unexplored. Here, we present the cellular effects of OPD on cell viability, proliferation, apoptosis, and metastasis in YD38 OSCC cells. OPD strongly inhibited the cell proliferation and metastasis, and induced apoptosis in YD38

Received May 24, 2024; **Revised** June 13, 2024; **Accepted** June 14, 2024
***Correspondence to:** Hoon Yoo, E-mail: hoon_yoo@chosun.ac.kr https://orcid.org/0000-0002-9249-1446

cells, suppressing vital proteins in the integrin/matrix metalloproteinases (MMPs)/FAK and AKT/PI3K/mTor pathways.

Materials and Methods

1. Chemicals and reagents

OPD (≥98% of purity) was purchased from Chengdu Biopurify Phytochemicals Co., Ltd.. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin were obtained from Sigma-Aldrich. BCA protein assay kit was purchased from Thermo Fisher Scientific. RPMI-1640 media, 10% fetal bovine serum (FBS), trypsin ethylene diamine tetraacetic acid and antibiotic-antimycotic solution were obtained from Welgene. Primary antibodies of cyclins, cdk6, poly ADP ribose polymerase (PARP), MMP-2, MMP-9, fibronectin, p-AKT, p-mTor, and beta actin were provided by Santa Cruz Biotechnology. Integrin α5, p-FAK, cleaved caspase-3, PI3Kβ, PI3K p85, p-PI3K, p-ERK, p-JNK, p-p38 were purchased from Cell Signaling Technology Inc.. Secondary antibodies were from Novus Biologicals.

2. Cell lines and cell culture

YD38 gingival cancer cells (including FaDu hypopharyngeal adenocarcinoma and YD15 tongue cancer) were purchased from Korean cell line bank. Cells were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and cultured in a humidified incubator with 5% CO_2 at 37℃.

3. MTT assay

Cell viability was measured by an MTT assay. Briefly, cells were seeded in 96-well plates (1×10^3 cells/well) and incubated overnight. OPD was treated at various concentrations and incubated for 24 hours or 48 hours at 37℃. After treatment, 10 μL of MTT (5 mg/mL) solution was added into each well and cells were further incubated at 37℃ for 4 hours. Finally, 100 μL of dimethyl sulfoxide was added to each well. The absorbance at 540 nm was measured in each well with a plate reader (Multiskan; Thermo Fisher Scientific).

4. Western blot analysis

The cells were seeded at the density of 5×10^5 cells/mL of medium in 100 mm culture dishes and incubated for 24 hours. Then, cells were treated with OPD (0, 50, 100, 150 and 200 μmol/L) for 48 hours. After that, whole cells were homogenized and the protein concentrations were determined using a BCA kit. The total proteins were separated by running on SDS-PAGE and transferred onto PVDF membrane (Merck Millipore). The PVDF membranes were blocked with 5% skim milk in PBS with Tween-20 for 1 hours and then incubated overnight at 4˚C with primary antibodies of target proteins. The primary antibodies and β-actin were diluted in 1:1,000 and 1:2,000, respectively. After washing with PBS-T (phosphate buffered saline containing 0.1% Tween-20) for 15 minutes, the membranes were incubated with corresponding secondary antibodies for 2 hours at room temperature. The signals were detected and analyzed by a Kodak Digital Science Image Station (Bruker BioSpin).

Results

1. OPD selectively exerted cytotoxic effects against YD38 cells

The effects of OPD on cell viability were determined by an MTT assay. YD38 cells treated with various concentrations of OPD (Fig. 1) for 48 hours significantly decreased the percent cell viability at the concentration above 75 μM. On contrary, cells of FaDu and YD15 were not affected by OPD, indicating that OPD selectively exerted cytotoxic effects against YD38 cells (Fig. 2).

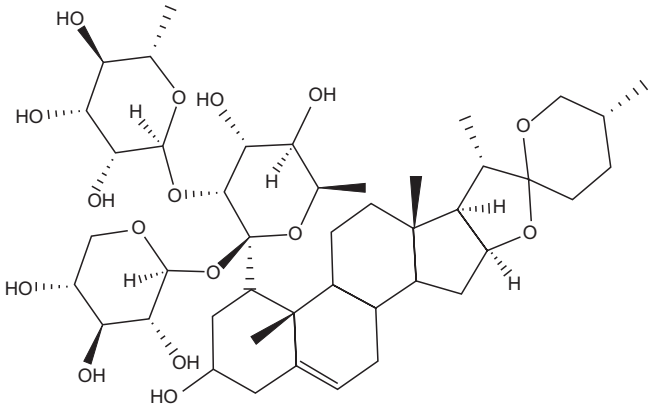

Fig. 1. Chemical structure of ophiopogonin D (OPD).

2. OPD downregulated the cell cycle regulators

The hyperactivation of cell cycle check points plays an essential role in regulating uncontrolled proliferation in various types of OSCC. In Western blotting results, the overexpressed cell cycle regulators of cyclin A, E, B1, D and cdk6 were dose-dependently suppressed by the treatment of OPD (Fig. 3).

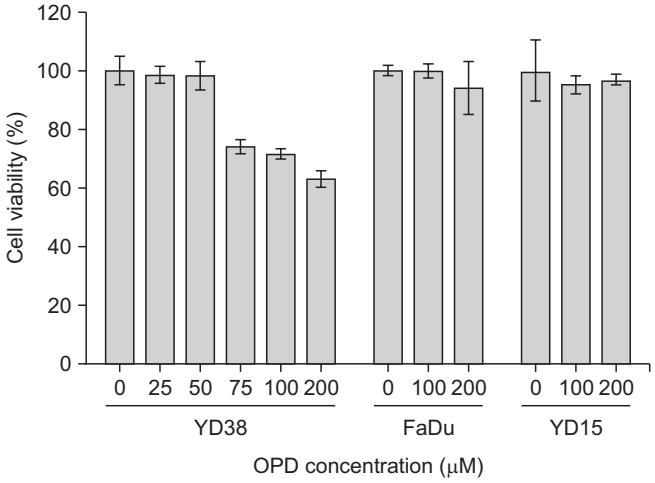

Fig. 2. The effect of OPD on oral cancer cells' viability. YD38, YD15 and FaDu cells were treated with various concentrations of OPD for 48 hours and the percent cell viability was determined by an MTT assay.

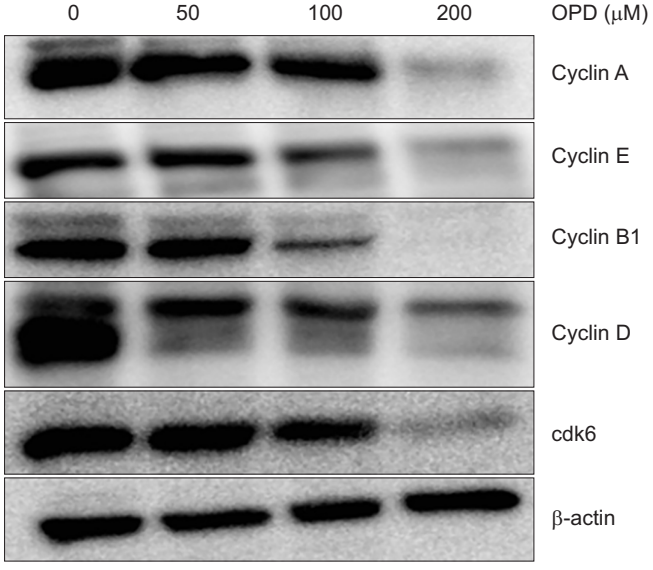

Fig. 3. OPD suppressed the expression of cell cycle regulators in YD38 cells. Cells (5×10^5 cells/well) were treated with OPD for 24 hours. Western blotting were carried out for the analysis of cyclin family and cdk6 expressions.

3. OPD induced apoptosis on YD38 cells

The effect of OPD on the programed cell death was investigated by detecting the expressions of apoptotic markers, cleaved caspase-3 and PARP (116 and 89 kDa). At the last phase of caspase-dependent apoptotic process, executioner cleaved caspase-3 propagates an apoptotic signal by the enzymatic action on downstream targets, including PARP and other substrates. As show in Fig. 4, OPD strongly activated caspase-3 and PARP dose-dependently with the increased expressions of cleaved caspase-3 and PARP. In addition, OPD dramatically increased the expression of light chain-3B (LC3B-II), a microtubule associated protein in autophagy induction, in a dose-dependent manner (Fig. 4).

4. OPD suppressed metastasis through integrin/MMPs/FAK pathway

The treatment with OPD significantly suppressed the expression of MMP enzymes (MMP-2 and MMP-9), which play significant roles in extracellular matrix remodeling and angiogenesis of tumor generation (Fig. 5). OPD also suppressed the upstream factors of fibronectin and integrin α5, and a downstream regulator of p-FAK, leading to the inhibition of cell adhesion, mobility, and metastasis.

5. OPD inhibited the PI3K/AKT/mTor and JNK pathways

We further investigated whether OPD could affect the signaling pathway of PI3K/AKT/mTor, a major oncogenic signal-

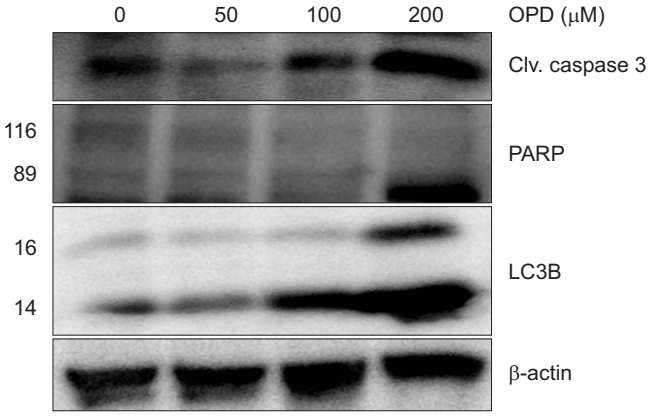

Fig. 4. The effect of OPD on apoptosis induction in YD38 cells. Cells (5×10^5 cells/well) were treated with OPD for 24 hours and cell lysates were subjected to Western blotting analysis.

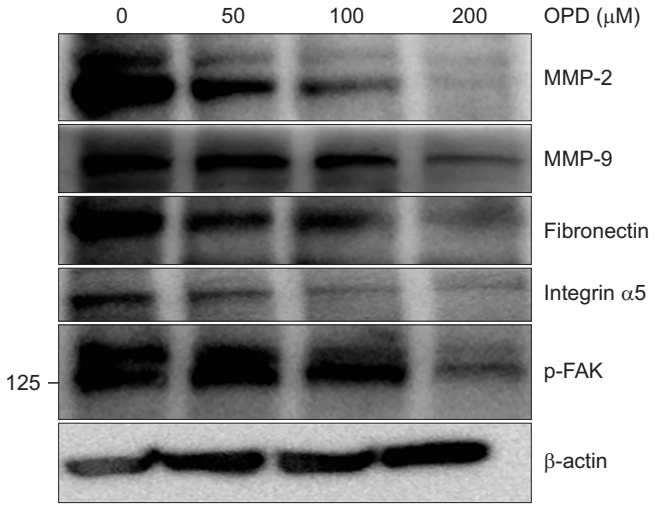

Fig. 5. OPD suppressed the metastasis of YD38 cells. Cells (5×10^5 cells/well) were treated with OPD (0–200 μM) for 24 hours. Western blotting was carried out by using antibodies of matrix metalloproteinase (MMP-2, MMP-9), fibronectin, integrin α5, p-FAK and β-actin.

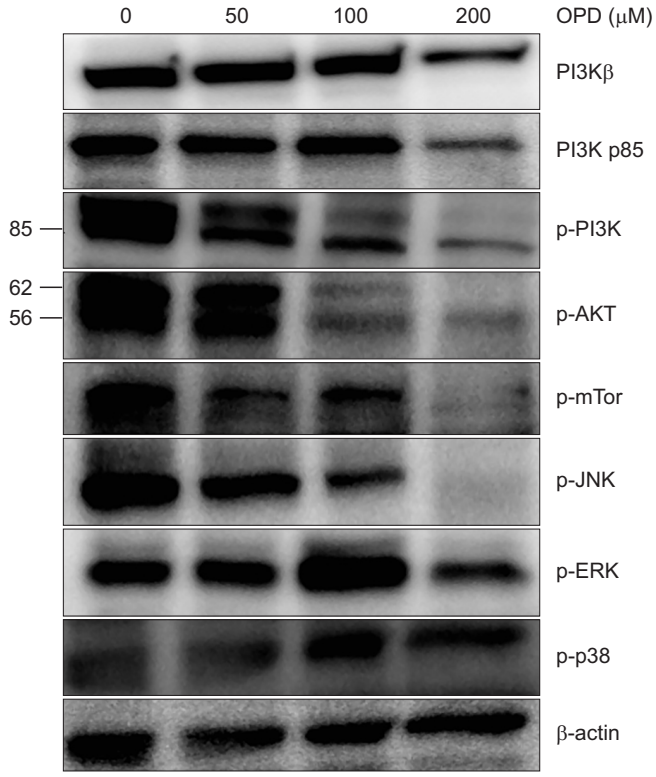

Fig. 6. OPD suppressed PI3Kβ and PI3K p85 as well as PI3K/AKT/mTor and c-Jun N-terminal kinase (JNK) pathways. Cells (5×10^5 cells/well) were treated with OPD (0–200 μM) for 24 hours. Cell lysates were subjected to western blotting for the expression analysis of PI3Kβ, PI3K p85, p-PI3K, p-AKT, p-mTor, p-JNK, p-ERK, p-p38 and β-actin.

ing cascade implicated in cell proliferation and survival. Our results showed that OPD suppressed the constitutive activation of PI3Kβ and PI3K p85 as well as the PI3K/AKT/mTor phosphorylation in a dose-dependent manner. In addition, in a MAPK signaling pathway, OPD significantly downregulated the phosphorylation of c-Jun N-terminal kinase (JNK) while less affecting the expressions of p-p38 and p-ERK (Fig. 6).

Discussion

In the present study, we investigated the cellular effects and mechanism of OPD on YD38 OSCC. OPD exhibited significant inhibitory effects on cell proliferation and potential cytotoxicity in YD38 cells by interrupting cell cycle and multiple cellular signaling pathways. Among three OSCC cell lines tested, YD38 gingival cancer cells showed the highest sensitivity to OPD in cell viability, suggesting that OPD has selective cytotoxicity towards YD38 cells. This could be beneficial for the treatment of gingival cancer, minimizing undesirable effects on other tissues. Cell cycle progression is regulated by a number of proteins in the cdk/cyclin family. The onset and progression of various types of cancers, including OSCC, are attributed to the overexpression and activation of these regulators [16-18]. Interestingly, in our results, the treatment of YD38 cells with OPD markedly reduced the expression of cyclin A, E, B1, and D, as well as cdk6, thereby inhibiting cell cycle progression. Cellular apoptosis is abnormally dysregulated at various stages in OSCC, particularly at the last phase of apoptosis when executioner caspases are activated to commit programmed cell death [3,19,20]. In this work, we confirmed OPD-induced apoptosis by detecting increased expression of both cleaved caspase-3 and cleaved PARP via Western blotting. This is consistent with an earlier report showing that OPD-induced apoptosis involved intrinsic and extrinsic apoptotic pathways in human breast cancer cells [21]. OPD also inhibited the expression of MMPs in YD38 cells, which play vital roles in the metastasis of cancer [22]. This inhibitory effect on metastasis was further confirmed by the inhibition of fibronectin, integrin α5, and FAK phosphorylation. Interestingly, the inhibition of MMP-2 and MMP-9 was associated with the strong suppression of p-JNK, not p-p38 or p-ERK, in YD38 cells. This suggests that the inhibitory effects of OPD on metastasis, as well as cytotoxic effect, might be mediated through the JNK pathway. In addition, OPD suppressed the PI3K/AKT/mTor pathway, an intracellular signaling pathway related to cellular quiescence, proliferation, and cancer. OPD's suppression of this pathway might have caused apoptosis induction as well as cell proliferation inhibition [23]. Most notably, the activation of PI3Kβ and

PI3K p85 was markedly suppressed by OPD. Considering that PI3K has been strongly implicated in oral tumorigenesis, as 7% of oral pre-cancer patient samples bear mutations in the PI3K gene [24], the anti-tumor effects of OPD on YD38 gingival cancer cells may have worked through the PI3K/AKT/mTor pathway.

In conclusion, OPD strongly inhibited cell proliferation and metastasis and induced apoptosis in YD38 cells. The cellular effects of OPD were associated with the inhibition of cell cycle regulators, caspase-dependent apoptosis, and cell mobility inhibition by downregulating the integrin/MMPs/FAK pathway and PI3K/AKT/mTor pathways. Thus, OPD could be an effective adjuvant for treating gingival cancer.

Funding

This study was supported by the National Research Foundation (NRF) of Korea funded by the Korea Government (No. RS-2023-00222390).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

References

1. Asmin PK, Nusrath F, Divakar DD. Occurrence and distribution of cancers with emphasis upon oral cancers in registered oncology institutes of South India – a retrospective study. Indian J Community Med 2024;49:120-30. doi: 10.4103/ijcm.ijcm_106_23
2. Badwelan M, Muaddi H, Ahmed A, Lee KT, Tran SD. Oral squamous cell carcinoma and concomitant primary tumors, what do we know? A review of the literature. Curr Oncol 2023;30:3721-34. doi: 10.3390/curroncol30040283
3. Tan Y, Wang Z, Xu M, Li B, Huang Z, Qin S, Nice EC, Tang J, Huang C. Oral squamous cell carcinomas: state of the field and emerging directions. Int J Oral Sci 2023;15:44. doi: 10.1038/s41368-023-00249-w
4. da Silva SD, Hier M, Mlynarek A, Kowalski LP, Alaoui-Jamali MA. Recurrent oral cancer: current and emerging therapeutic approaches. Front Pharmacol 2012;3:149. doi: 10.3389/fphar.2012.00149
5. Zhang W, Jing Y, Wang S, Wu Y, Sun Y, Zhuang J, Huang X, Chen S, Zhang X, Song Y, Hu Q, Ni Y. Identification of biological functions and prognostic value of NNMT in oral squamous cell carcinoma. Biomolecules 2022;12:1487. doi: 10.3390/biom12101487
6. Chen MH, Chen XJ, Wang M, Lin LG, Wang YT. Ophiopogon japonicus--a phytochemical, ethnomedicinal and pharmacological review. J Ethnopharmacol 2016;181:193-213. doi: 10.1016/j.jep.2016.01.037
7. Xu J, Wang Y, Xu DS, Ruan KF, Feng Y, Wang S. Hypoglycemic effects of MDG-1, a polysaccharide derived from Ophiopogon japonicas, in the ob/ob mouse model of type 2 diabetes mellitus. Int J Biol Macromol 2011;49:657-62. doi: 10.1016/j.ijbiomac.2011.06.026
8. Lan S, Yi F, Shuang L, Chenjie W, Zheng XW. Chemical constituents from the fibrous root of Ophiopogon japonicus, and their effect on tube formation in human myocardial microvascular endothelial cells. Fitoterapia 2013;85:57-63. doi: 10.1016/j.fitote.2012.12.025
9. Chen YT, Yuan Q, Shan LT, Lin MA, Cheng DQ, Li CY. Antitumor activity of bacterial exopolysaccharides from the endophyte Bacillus amyloliquefaciens sp. isolated from Ophiopogon japonicus. Oncol Lett 2013;5:1787-92. doi: 10.3892/ol.2013.1284
10. Zheng Q, Feng Y, Xu DS, Lin X, Chen YZ. Influence of sulfation on anti-myocardial ischemic activity of Ophiopogon japonicus polysaccharide. J Asian Nat Prod Res 2009;11:306-21. doi: 10.1080/10286020902727363
11. Wang X, Wang T, Ding S, Ma YL. Xin Su Ning-a review of basic and clinical pharmacology integrated with traditional Chinese medicine antiarrhythmic theory. Front Pharmacol 2021;12:657484. doi: 10.3389/fphar.2021.657484
12. Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981-2002. J Nat Prod 2003;66:1022-37. doi: 10.1021/np030096l
13. Kou J, Tian Y, Tang Y, Yan J, Yu B. Antithrombotic activities of aqueous extract from Radix Ophiopogon japonicus and its two constituents. Biol Pharm Bull 2006;29:1267-70. doi: 10.1248/bpb.29.1267
14. Kou J, Sun Y, Lin Y, Cheng Z, Zheng W, Yu B, Xu Q. Anti-inflammatory activities of aqueous extract from Radix Ophiopogon japonicus and its two constituents. Biol Pharm Bull 2005;28:1234-8. doi: 10.1248/bpb.28.1234
15. Qian J, Jiang F, Wang B, Yu Y, Zhang X, Yin Z, Liu C. Ophiopogonin D prevents H2O2-induced injury in primary hu-

man umbilical vein endothelial cells. J Ethnopharmacol 2010;128:438–45. doi: 10.1016/j.jep.2010.01.031
16. Ding L, Cao J, Lin W, Chen H, Xiong X, Ao H, Yu M, Lin J, Cui Q. The roles of cyclin-dependent kinases in cell-cycle progression and therapeutic strategies in human breast cancer. Int J Mol Sci 2020;21:1960. doi: 10.3390/ijms21061960
17. Casimiro MC, Crosariol M, Loro E, Li Z, Pestell RG. Cyclins and cell cycle control in cancer and disease. Genes Cancer 2012;3:649–57. doi: 10.1177/1947601913479022
18. Hu Q, Huang T. Regulation of the cell cycle by ncRNAs affects the efficiency of CDK4/6 inhibition. Int J Mol Sci 2023;24:8939. doi: 10.3390/ijms24108939
19. Vitale I, Pietrocola F, Guilbaud E, Aaronson SA, Abrams JM, Adam D, Agostini M, Agostinis P, Alnemri ES, Altucci L, Amelio I, Andrews DW, Aqeilan RI, Arama E, Baehrecke EH, Balachandran S, Bano D, Barlev NA, Bartek J, Bazan NG, Becker C, Bernassola F, Bertrand MJM, Bianchi ME, Blagosklonny MV, Blander JM, Blandino G, Blomgren K, Borner C, Bortner CD, Bove P, Boya P, Brenner C, Broz P, Brunner T, Damgaard RB, Calin GA, Campanella M, Candi E, Carbone M, Carmona-Gutierrez D, Cecconi F, Chan FK, Chen GQ, Chen Q, Chen YH, Cheng EH, Chipuk JE, Cidlowski JA, Ciechanover A, Ciliberto G, Conrad M, Cubillos-Ruiz JR, Czabotar PE, D' Angiolella V, Daugaard M, Dawson TM, Dawson VL, De Maria R, De Strooper B, Debatin KM, Deberardinis RJ, Degterev A, Del Sal G, Deshmukh M, Di Virgilio F, Diederich M, Dixon SJ, Dynlacht BD, El-Deiry WS, Elrod JW, Engeland K, Fimia GM, Galassi C, Ganini C, Garcia-Saez AJ, Garg AD, Garrido C, Gavathiotis E, Gerlic M, Ghosh S, Green DR, Greene LA, Gronemeyer H, Häcker G, Hajnóczky G, Hardwick JM, Haupt Y, He S, Heery DM, Hengartner MO, Hetz C, Hildeman DA, Ichijo H, Inoue S, Jäättelä M, Janic A, Joseph B, Jost PJ, Kanneganti TD, Karin M, Kashkar H, Kaufmann T, Kelly GL, Kepp O, Kimchi A, Kitsis RN, Klionsky DJ, Kluck R, Krysko DV, Kulms D, Kumar S, Lavandero S, Lavrik IN, Lemasters JJ, Liccardi G, Linkermann A, Lipton SA, Lockshin RA, López-Otín C, Luedde T, MacFarlane M, Madeo F, Malorni W, Manic G, Mantovani R, Marchi S, Marine JC, Martin SJ, Martinou JC, Mastroberardino PG, Medema JP, Mehlen P, Meier P, Melino G, Melino S, Miao EA, Moll UM, Muñoz-Pinedo C, Murphy DJ, Niklison-Chirou MV, Novelli F, Núñez G, Oberst A, Ofengeim D, Opferman JT, Oren M, Pagano M, Panaretakis T, Pasparakis M, Penninger JM, Pentimalli F, Pereira DM, Pervaiz S, Peter ME, Pinton P, Porta G, Prehn JHM, Puthalakath H, Rabinovich GA, Rajalingam K, Ravichandran KS, Rehm M, Ricci JE, Rizzuto R, Robinson N, Rodrigues CMP, Rotblat B, Rothlin CV, Rubinsztein DC, Rudel T, Rufini A, Ryan KM, Sarosiek KA, Sawa A, Sayan E, Schroder K, Scorrano L, Sesti F, Shao F, Shi Y, Sica GS, Silke J, Simon HU, Sistigu A, Stephanou A, Stockwell BR, Strapazzon F, Strasser A, Sun L, Sun E, Sun Q, Szabadkai G, Tait SWG, Tang D, Tavernarakis N, Troy CM, Turk B, Urbano N, Vandenabeele P, Vanden Berghe T, Vander Heiden MG, Vanderluit JL, Verkhratsky A, Villunger A, von Karstedt S, Voss AK, Vousden KH, Vucic D, Vuri D, Wagner EF, Walczak H, Wallach D, Wang R, Wang Y, Weber A, Wood W, Yamazaki T, Yang HT, Zakeri Z, Zawacka-Pankau JE, Zhang L, Zhang H, Zhivotovsky B, Zhou W, Piacentini M, Kroemer G, Galluzzi L. Apoptotic cell death in disease-current understanding of the NCCD 2023. Cell Death Differ 2023;30:1097–154. doi: 10.1038/s41418-023-01153-w
20. He S, Chakraborty R, Ranganathan S. Proliferation and apoptosis pathways and factors in oral squamous cell carcinoma. Int J Mol Sci 2022;23:1562. doi: 10.3390/ijms23031562
21. Zang QQ, Zhang L, Gao N, Huang C. Ophiopogonin D inhibits cell proliferation, causes cell cycle arrest at G2/M, and induces apoptosis in human breast carcinoma MCF-7 cells. J Integr Med 2016;14:51–9. doi: 10.1016/S2095-4964(16)60238-8
22. Zhang Y, Han Y, Zhai K, Sun M, Liu J, Yu B, Kou J. Ophiopogonin-D suppresses MDA-MB-435 cell adhesion and invasion by inhibiting matrix metalloproteinase-9. Mol Med Rep 2015;12:1493–8. doi: 10.3892/mmr.2015.3541
23. Zhang YY, Meng C, Zhang XM, Yuan CH, Wen MD, Chen Z, Dong DC, Gao YH, Liu C, Zhang Z. Ophiopogonin D attenuates doxorubicin-induced autophagic cell death by relieving mitochondrial damage in vitro and in vivo. J Pharmacol Exp Ther 2015;352:166–74. doi: 10.1124/jpet.114.219261
24. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. Oncogene 2008;27:5497–510. doi: 10.1038/onc.2008.245